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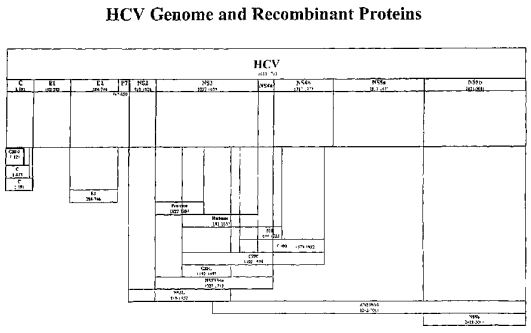
(54)【発明の名称】ＨＣＶ抗原／抗体の組合せアッセイ

(57)【要約】
本発明によって、ＨＣＶコア抗原およびＮＳ３／４ａ抗体の組合せアッセイ（ｃｏｍｂｉｎａｔｉｏｎ　ａｓｓａｙ）が提供される。このＨＣＶコア抗原およびＮＳ３／４ａ抗体の組合せアッセイは、単一の固体マトリクスを用いて、サンプル中に存在するＨＣＶ抗原およびＨＣＶ抗体の両方を検出し得る。さらに、本発明によって、このＨＣＶコア抗原およびＮＳ３／４ａ抗体の組合せアッセイにおいて用いるためのイムノアッセイ固体支持体もまた、提供される。



【特許請求の範囲】
【請求項１】
イムノアッセイ固体支持体であって、該支持体に結合された、少なくとも１つのＣ型肝炎ウイルス（ＨＣＶ）抗コア抗体、および少なくとも１つの単離されたＨＣＶ　ＮＳ３／４ａエピトープを含む、支持体。
【請求項２】
前記支持体に結合された少なくとも２つのＨＣＶ抗コア抗体を含む、請求項１に記載のイムノアッセイ固体支持体。
【請求項３】
前記少なくとも１つの抗コア抗体が、ＨＣＶコア抗原のＮ末端領域に対して指向される、請求項１に記載のイムノアッセイ固体支持体。
【請求項４】
前記少なくとも１つの抗コア抗体が、ＨＣＶ１ポリタンパク質配列に対して番号付された、ＨＣＶのアミノ酸１０～５３に対して指向される、請求項３に記載のイムノアッセイ固体支持体。
【請求項５】
前記少なくとも１つの抗コア抗体が、モノクローナル抗体である、請求項１に記載のイムノアッセイ固体支持体。
【請求項６】
前記ＮＳ３／４ａエピトープが、コンホメーションエピトープであり、そして図４Ａ～４Ｄに示されるアミノ酸配列を含む、請求項１に記載のイムノアッセイ固体支持体。
【請求項７】
前記支持体に結合された多エピトープ融合抗原をさらに含む、請求項１に記載のイムノアッセイ固体支持体。
【請求項８】
前記多エピトープ融合抗原が、図７Ａ～７Ｆに示されるアミノ酸配列を含む、請求項７に記載のイムノアッセイ固体支持体。
【請求項９】
イムノアッセイ固体支持体であって、該支持体に結合された、２つのＣ型肝炎ウイルス（ＨＣＶ）抗コアモノクローナル抗体、および図４Ａ～４Ｄに示されるアミノ酸配列を含むＨＣＶ　ＮＳ３／４ａコンホメーションエピトープを含む、支持体。
【請求項１０】
前記２つの抗コア抗体が、ＨＣＶコア抗原のＮ末端領域に対して指向される、請求項９に記載のイムノアッセイ固体支持体。
【請求項１１】
前記２つの抗コア抗体が、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１０～５３に対して指向される、請求項１０に記載のイムノアッセイ固体支持体。
【請求項１２】
イムノアッセイ固体支持体であって、該支持体に結合された、２つのＣ型肝炎ウイルス（ＨＣＶ）抗コアモノクローナル抗体、図４Ａ～４Ｄに示されるアミノ酸配列を含むＨＣＶ　ＮＳ３／４ａコンホメーションエピトープ、および図７Ａ～７Ｆに示されるアミノ酸配列を含む多エピトープ融合抗原を含む、支持体。
【請求項１３】
生物学的サンプルにおけるＣ型肝炎ウイルス（ＨＣＶ）感染を検出する方法であって、該方法は、以下：
（ａ）　請求項１に記載のイムノアッセイ固体支持体を提供する工程；
（ｂ）　生物学的サンプルと該固体支持体を、ＨＣＶ抗原および抗体が、該生物学的サンプル中に存在する場合に、それぞれ、前記少なくとも１つの抗コア抗体およびＮＳ３／４ａエピトープに結合可能な条件下で混合する工程；

（ｃ）　以下を、工程（ｂ）由来の固体支持体に、複合体を形成する条件下で加える工程：
（ｉ）　第１の検出可能な標識抗体であって、ここで、該第１の検出可能な標識抗体は、検出可能な標識ＨＣＶ抗コア抗体であり、ここで、該標識抗コア抗体は、該固体支持体に結合した少なくとも１つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される、第１の検出可能な標識抗体；
（ｉｉ）　該ＮＳ３／４ａエピトープと反応する生物学的サンプル由来のＨＣＶ抗体と反応する抗原；および
（ｉｉｉ）　（ｉｉ）の抗原と反応する、第２の検出可能な標識抗体；
（ｄ）　もしあれば、該生物学的サンプルにおけるＨＣＶ感染を示すような、該抗体と該抗原との間に形成される複合体を検出する工程、
を包含する、方法。
【請求項１４】
前記少なくとも１つの抗コア抗体は、前記ＨＣＶコア抗原のＮ末端領域に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、該ＨＣＶコア抗原のＣ末端領域に対して指向される、請求項１３に記載の方法。
【請求項１５】
前記少なくとも１つの抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１０～５３に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１２０～１３０に対して指向される、請求項１４に記載の方法。
【請求項１６】
前記生物学的サンプル由来のＨＣＶ抗体と反応する前記抗原が、前記ＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープを含む、請求項１３に記載の方法。
【請求項１７】
前記ｃ３３ｃエピトープが、ヒトスーパーオキシドジスムターゼ（ｈＳＯＤ）アミノ酸配列と融合され、そして前記第２の検出可能な標識抗体が、該ｈＳＯＤアミノ酸配列と反応する、請求項１６に記載の方法。
【請求項１８】
前記ＮＳ３／４ａエピトープが、コンホメーションエピトープであり、そして図４Ａ～４Ｄに示されるアミノ酸配列を含む、請求項１３に記載の方法。
【請求項１９】
生物学的サンプルにおけるＣ型肝炎ウイルス（ＨＣＶ）感染を検出する方法であって、該方法は、以下：
（ａ）　請求項２に記載のイムノアッセイ固体支持体を提供する工程；
（ｂ）　生物学的サンプルと該固体支持体を、ＨＣＶ抗原および抗体が、該生物学的サンプル中に存在する場合に、それぞれ、前記少なくとも２つの抗コア抗体およびＮＳ３／４ａエピトープに結合可能な条件下で混合する工程；
（ｃ）　以下を、工程（ｂ）由来の固体支持体に、複合体を形成する条件下で加える工程：
（ｉ）　第１の検出可能な標識抗体であって、ここで、該第１の検出可能な標識抗体は、検出可能な標識ＨＣＶ抗コア抗体であり、ここで、該標識抗コア抗体は、該固体支持体に結合した少なくとも２つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される、第１の検出可能な標識抗体；
（ｉｉ）　ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ；および
（ｉｉｉ）　該ｈＳＯＤアミノ酸配列と反応する、第２の検出可能な標識抗体；
（ｄ）　もしあれば、該生物学的サンプルにおけるＨＣＶ感染を示すような、該抗体と該抗原との間に形成される複合体を検出する工程、
を包含する、方法。

【請求項２０】
前記ＮＳ３／４ａエピトープが、コンホメーションエピトープであり、そして図４Ａ～４Ｄに示されるアミノ酸配列を含む、請求項１９に記載の方法。
【請求項２１】
生物学的サンプルにおけるＣ型肝炎ウイルス（ＨＣＶ）感染を検出する方法であって、該方法は、以下：
（ａ）　請求項９に記載のイムノアッセイ固体支持体を提供する工程；
（ｂ）　生物学的サンプルと該固体支持体を、ＨＣＶ抗原および抗体が、該生物学的サンプル中に存在する場合に、それぞれ、前記少なくとも２つの抗コア抗体およびＮＳ３／４ａコンホメーションエピトープに結合可能な条件下で混合する工程；
（ｃ）　以下を、工程（ｂ）由来の固体支持体に、複合体を形成する条件下で加える工程：
（ｉ）　第１の検出可能な標識抗体であって、ここで、該第１の検出可能な標識抗体は、検出可能な標識ＨＣＶ抗コア抗体であり、ここで、該標識抗コア抗体は、該固体支持体に結合した少なくとも２つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される、第１の検出可能な標識抗体；
（ｉｉ）　ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ；および
（ｉｉｉ）　該ｈＳＯＤアミノ酸配列と反応する、第２の検出可能な標識抗体；
（ｄ）　もしあれば、該生物学的サンプルにおけるＨＣＶ感染を示すような、該抗体と該抗原との間に形成される複合体を検出する工程、
を包含する、方法。
【請求項２２】
前記少なくとも２つの抗コア抗体は、前記ＨＣＶコア抗原のＮ末端領域に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、該ＨＣＶコア抗原のＣ末端領域に対して指向される、請求項２１に記載の方法。
【請求項２３】
前記少なくとも２つの抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１０～５３に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、該ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１２０～１３０に対して指向される、請求項２２に記載の方法。
【請求項２４】
生物学的サンプルにおけるＣ型肝炎ウイルス（ＨＣＶ）感染を検出する方法であって、該方法は、以下：
（ａ）　請求項７に記載のイムノアッセイ固体支持体を提供する工程；
（ｂ）　生物学的サンプルと該固体支持体を、ＨＣＶ抗原および抗体が、該生物学的サンプル中に存在する場合に、前記少なくとも１つの抗コア抗体、ＮＳ３／４ａエピトープ、および多エピトープ融合抗原に結合可能な条件下で混合する工程；
（ｃ）　以下を、工程（ｂ）由来の固体支持体に、複合体を形成する条件下で加える工程：
（ｉ）　第１の検出可能な標識抗体であって、ここで、該第１の検出可能な標識抗体は、検出可能な標識ＨＣＶ抗コア抗体であり、ここで、該標識抗コア抗体は、該固体支持体に結合した少なくとも１つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される、第１の検出可能な標識抗体；
（ｉｉ）　第１の抗原および第２の抗原であって、該抗原は、それぞれ、該ＮＳ３／４ａエピトープおよび該多エピトープ融合抗原に反応する生物学的サンプル由来のＨＣＶ抗体と反応する、第１の抗原および第２の抗原；および
（ｉｉｉ）　（ｉｉ）の抗原と反応する、第２の検出可能な標識抗体；
（ｄ）　もしあれば、該生物学的サンプルにおけるＨＣＶ感染を示すような、該抗体と該抗原との間に形成される複合体を検出する工程、

を包含する、方法。
【請求項２５】
前記少なくとも１つの抗コア抗体は、前記ＨＣＶコア抗原のＮ末端領域に対して指向され、そして該第１の検出可能な標識ＨＣＶ抗コア抗体は、該ＨＣＶコア抗原のＣ末端領域に対して指向される、請求項２４に記載の方法。
【請求項２６】
前記少なくとも１つの抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１０～５３に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１２０～１３０に対して指向される、請求項２５に記載の方法。
【請求項２７】
前記生物学的サンプル由来のＨＣＶ抗体と反応する前記第１の抗原が、前記ＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープを含む、請求項２４に記載の方法。
【請求項２８】
前記ｃ３３ｃエピトープが、ヒトスーパーオキシドジスムターゼ（ｈＳＯＤ）アミノ酸配列と融合され、そして前記第２の検出可能な標識抗体が、該ｈＳＯＤアミノ酸配列と反応する、請求項２７に記載の方法。
【請求項２９】
前記生物学的サンプル由来のＨＣＶ抗体と反応する前記第２の抗原が、前記ＨＣＶポリタンパク質のｃ２２領域由来のエピトープを含む、請求項２４に記載の方法。
【請求項３０】
前記ｃ２２領域由来のエピトープは、前記ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶポリタンパク質のアミノ酸Lys_{10}～Ser_{99}を含み、該アミノ酸Lys_{10}～Ser_{99}は、Ａｒｇ４７の欠失および４４位でのＴｒｐのＬｅｕへの置換を有し、ここで、該エピトープは、ヒトスーパーオキシドジスムターゼ（ｈＳＯＤ）アミノ酸配列と融合され、そして前記第２の検出可能な標識抗体は、該ｈＳＯＤアミノ酸配列と反応する、請求項２９に記載の方法。
【請求項３１】
前記多エピトープ融合抗原が、図７Ａ～７Ｆに示されるアミノ酸配列を含む、請求項２４に記載の方法。
【請求項３２】
生物学的サンプルにおけるＣ型肝炎ウイルス（ＨＣＶ）感染を検出する方法であって、該方法は、以下：
（ａ）　請求項１２に記載のイムノアッセイ固体支持体を提供する工程；
（ｂ）　生物学的サンプルと該固体支持体を、ＨＣＶ抗原および抗体が、該生物学的サンプル中に存在する場合に、それぞれ、前記少なくとも２つの抗コア抗体、ＮＳ３／４ａコンホメーションエピトープ、および多エピトープ融合抗原に結合可能な条件下で混合する工程；
（ｃ）　以下を、工程（ｂ）由来の固体支持体に、複合体を形成する条件下で加える工程：
（ｉ）　第１の検出可能な標識抗体であって、ここで、該第１の検出可能な標識抗体は、検出可能な標識ＨＣＶ抗コア抗体であり、ここで、該標識抗コア抗体は、該固体支持体に結合した少なくとも２つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される、第１の検出可能な標識抗体；
（ｉｉ）　ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ、およびｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ２２領域由来のエピトープ；および
（ｉｉｉ）　該ｈＳＯＤアミノ酸配列と反応する、第２の検出可能な標識抗体；
（ｄ）　もしあれば、該生物学的サンプルにおけるＨＣＶ感染を示すような、該抗体と該抗原との間に形成される複合体を検出する工程、

を包含する、方法。
【請求項３３】
前記少なくとも２つの抗コア抗体は、前記ＨＣＶコア抗原のＮ末端領域に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、該ＨＣＶコア抗原のＣ末端領域に対して指向される、請求項３２に記載の方法。
【請求項３４】
前記少なくとも２つの抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１０～５３に対して指向され、そして前記検出可能な標識ＨＣＶ抗コア抗体は、ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶのアミノ酸１２０～１３０に対して指向される、請求項３３に記載の方法。
【請求項３５】
前記ｃ２２領域由来のエピトープは、前記ＨＣＶ１ポリタンパク質配列に対して番号付けした、ＨＣＶポリタンパク質のアミノ酸Lys_{10}～Ser_{99}を含み、該アミノ酸Lys_{10}～Ser_{99}は、Ａｒｇ４７の欠失および４４位でのＴｒｐのＬｅｕへの置換を有する、請求項３２に記載の方法。
【請求項３６】
免疫診断的検査キットであって、請求項１～１２のいずれかに１項に記載のイムノアッセイ固体支持体、および免疫診断的検査を行うための指示書を含む、キット。
【請求項３７】
イムノアッセイ固体支持体を作製する方法であって、該方法は、以下：
（ａ）　固体支持体を提供する工程；および
（ｂ）　少なくとも１つのＣ型肝炎ウイルス（ＨＣＶ）抗コア抗体、および少なくとも１つの単離されたＨＣＶ　ＮＳ３／４ａコンホメーションエピトープを、該支持体に対して結合させる工程、
を包含する、方法。
【請求項３８】
イムノアッセイ固体支持体を作製する方法であって、該方法は、以下：
（ａ）　固体支持体を提供する工程；および
（ｂ）　２つのＣ型肝炎ウイルス（ＨＣＶ）抗コア抗体、および単離されたＨＣＶ　ＮＳ３／４ａコンホメーションエピトープを、該支持体に対して結合させる工程、
を包含する、方法。
【請求項３９】
多エピトープ融合抗原の少なくとも１つを、前記支持体に結合させる工程をさらに包含する、請求項３７または３８に記載の方法。
【請求項４０】
多エピトープ融合抗原であって、図７Ａ～７Ｆに示されるアミノ酸配列、または図７Ａ～７Ｆに示されるアミノ酸配列に対して少なくとも８０％の配列同一性を有するアミノ酸配列を含み、ＨＣＶに感染した個体由来の生物学的サンプルに存在する抗ＨＣＶ抗体と特異的に反応する、多エピトープ融合抗原。
【請求項４１】
請求項４０に記載の多エピトープ融合抗原であって、該多エピトープ融合抗原が、図７Ａ～７Ｆに示されるアミノ酸配列、または図７Ａ～７Ｆに示されるアミノ酸配列に対して少なくとも９０％の配列同一性を有するアミノ酸配列を含み、ＨＣＶに感染した個体由来の生物学的サンプルに存在する抗ＨＣＶ抗体と特異的に反応する、多エピトープ融合抗原。
【請求項４２】
前記多エピトープ融合抗原が、図５Ａ～５Ｆに示されるアミノ酸配列からなる、請求項４０に記載の多エピトープ融合抗原。
【請求項４３】
請求項４０～４２のいずれか１項に記載の多エピトープ融合抗原についてのコード配列を含む、ポリヌクレオチド。

【請求項４４】
組換えベクターであって、以下：
（ａ）　請求項４３に記載のポリヌクレオチド；
（ｂ）　および、該ポリヌクレオチドに作動可能に連結された制御エレメントであって、これにより、コード配列は、宿主細胞において転写および翻訳され得る、制御エレメント、
を含む、組換えベクター。
【請求項４５】
請求項４４に記載の組換えベクターを用いて形質転換した、宿主細胞。
【請求項４６】
組換えの多エピトープ融合抗原を産生する方法であって、以下：
（ａ）　請求項４５に記載の宿主細胞の集団を提供する工程；および
（ｂ）　前記組換えベクター中に存在するコード配列によりコードされる該多エピトープ融合抗原が発現される条件下で、該細胞の集団を培養する工程、
を包含する、方法。
【発明の詳細な説明】
【０００１】
（技術分野）
本発明は、一般的にウイルスの診断に関連する。特に、本発明は、Ｃ型肝炎ウイルス感染を正確に診断するための抗原／抗体の組み合わせアッセイに関連する。
【０００２】
（発明の背景）
Ｃ型肝炎ウイルス（ＨＣＶ）は、主に輸血および性的接触を介して感染する、非経口の非Ａ型肝炎、非Ｂ型肝炎（ＮＡＮＢＨ）の主な原因である。このウイルスは、献血者の０．４～２．０％に存在する。慢性肝炎は、感染者の約５０％で発症し、そしてこれらの感染した個体のうちおよそ２０％が、肝硬変を発症し、これはしばしば肝細胞癌を引き起こす。従って、この疾患の研究および制御は、医学的に重要である。
【０００３】
ＨＣＶは、ＨｏｕｇｈｔｅｎらによってＮＡＮＢＨの原因として、最初に同定され、そして特徴付けされた。このＨＣＶのウイルスゲノム配列を得るための方法が公知であるのと同様に、この配列も公知である。例えば、国際公開番号ＷＯ８９／０４６６９；同ＷＯ９０／１１０８９；および同ＷＯ９０／１４４３６を参照のこと。ＨＣＶは、９．５ｋｂのポジティブセンス、単鎖ＲＮＡゲノムを有し、Ｆｌａｖｉｒｉｄａｅファミリーウイルスのメンバーである。系統発生分析に基づいて、少なくとも６つの異なる関連ＨＣＶの遺伝子が、同定されている（Ｓｉｍｍｏｎｄｓら、Ｊ．Ｇｅｎ．Ｖｉｒｏｌ．（１９９３）７４：２３９１～２３９９）。これらのウイルスは、３０００を超えるアミノ酸残基を有する単一のポリタンパク質をコードする（Ｃｈｏｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３５９～３６９；Ｃｈｏｏら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９１）８８：２４５１～２４５５；Ｈａｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９１）８８：１７１１～１７１５）。ポリタンパク質は、翻訳と同時に、そして翻訳後に、構造タンパク質および非構造（ＮＳ）タンパク質の両方にプロセシングされる。
【０００４】
特に、図１に示されるように、いくつかのタンパク質は、ＨＣＶゲノムによってコードされる。ＨＣＶポリタンパク質の切断生成物の順番および学名は以下である：NH_2－Ｃ－Ｅ１－Ｅ２－ｐ７－ＮＳ２－ＮＳ３－ＮＳ４ａ－ＮＳ４ｂ－ＮＳ５ａ－ＮＳ５ｂ－ＣＯＯＨ。ポリタンパク質の最初の切断は、３つの構造タンパク質（Ｎ末端ヌクレオカプシドタンパク質（「コア」と呼ばれる）ならびに２つのエンベロープ糖タンパク質（「Ｅ１」（Ｅとしても公知）および「Ｅ２」（Ｅ２／ＮＳ１としても公知））ならびにウイルス酵素を含む非構造（ＮＳ）タンパク質を遊離する、宿主プロテアーゼによって触媒される。

ＮＳ領域は、ＮＳ２、ＮＳ３、ＮＳ４、およびＮＳ５と呼ばれる。ＮＳ２は、タンパク質分解活性を有する内在性膜タンパク質である。ＮＳ２は、単独でか、またはＮＳ３と組み合わせてのいずれかで、ＮＳ２－ＮＳ３シスル（ｓｉｓｓｌｅ）結合を切断し、これは次にＮＳ３のＮ末端を生じ、セリンプロテアーゼおよびＲＮＡヘリカーゼの両方の活性を含む大きなポリタンパク質を放出する。ＮＳ３プロテアーゼは、残りのポリタンパク質をプロセシングするために役立つ。ポリタンパク質成熟の終了は、ＮＳ３－ＮＳ４ａの連結部での自己触媒的切断（ＮＳ３セリンプロテアーゼによって触媒される）によって開始される。引続くＨＣＶポリタンパク質のＮＳ３媒介切断は、別のポリペプチドのＮＳ３分子によるポリタンパク質切断連結部の認識に関連するようである。これらの反応において、ＮＳ３は、ＮＳ３補因子（ＮＳ４ａ）、２つのタンパク質（ＮＳ４ｂおよびＮＳ５ａ）、ならびにＲＮＡ依存性ＲＮＡポリメラーゼ（ＮＳ５ｂ）を遊離する。

【０００５】

ＨＣＶについての免疫学的試薬および診断的試薬として有用なＨＣＶポリタンパク質由来の多数の一般的なポリペプチドならびに特定のポリペプチドが記載されている。例えば、Ｈｏｕｇｈｔｏｎら、欧州公開番号３１８，２１６および同３８８，２３２；Ｃｈｏｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３５９～３６２；Ｋｕｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３６２～３６４；Ｈｏｕｇｈｔｏｎら、Ｈｅｐａｔｏｌｏｇｙ（１９９１）１４：３８１～３８８；Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９２）８９：１００１１～１００１５；Ｃｈｉｅｎら、Ｊ．Ｇａｓｔｒｏｅｎｔ．Ｈｅｐａｔｏｌ．（１９９３）８：Ｓ３３～３９；Ｃｈｉｅｎら、国際公開番号ＷＯ９３／００３６５；Ｃｈｉｅｎ，Ｄ．Ｙ．、国際公開番号ＷＯ９４／０１７７８を参照のこと。これらの刊行物は、一般的に、ＨＣＶならびにＨＣＶポリペプチドの免疫学的試薬の製造および使用についての広範な背景を提供する。

【０００６】

ＨＣＶ保有者およびＨＣＶに汚染された血液または血液製剤をスクリーニングおよび同定するための高感度で特異的な方法は、医薬品における重要な進歩を提供する。輸血後肝炎（ｐｏｓｔ－ｔｒａｎｓｆｕｓｉｏｎ ｈｅｐａｔｉｔｉｓ）（ＰＴＨ）は、輸血された患者のおよそ１０％で発症し、そしてＨＣＶは、これらの症例の最大９０％の原因であった。患者の看護についての、ならびに血液および血液製剤によるか、または緊密な個人的接触によるＨＣＶの予防および感染についての、確実な診断手段および予後診断手段が必要とされる。従って、いくつかのアッセイがＨＣＶ感染の血清診断のために開発されてきた。例えば、Ｃｈｏｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３５９～３６２；Ｋｕｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３６２～３６４；Ｃｈｏｏら、Ｂｒ．Ｍｅｄ．Ｂｕｌｌ．（１９９０）４６：４２３～４４１；Ｅｂｅｌｉｎｇら、Ｌａｎｃｅｔ（１９９０）３３５：９８２～９８３；ｖａｎ ｄｅｒ Ｐｏｅｌら、Ｌａｎｃｅｔ（１９９０）３３５：５５８～５６０；ｖａｎ ｄｅｒ Ｐｏｅｌら、Ｌａｎｃｅｔ（１９９１）３３７：３１７～３１９；Ｃｈｉｅｎ，Ｄ．Ｙ．、国際公開番号ＷＯ９４／０１７７８；Ｖａｌｅｎｚｕｅｌａら、国際公開番号ＷＯ９７／４４４６９；ならびにＫａｓｈｉｗａｋｕｍａら、米国特許第５，８７１，９０４号を参照のこと。

【０００７】

血清に基づくいくつかのアッセイで遭遇する重大な問題は、ウイルスの感染と検出との間に有意な間隔（しばしば、８０日間を超える）が存在することである。このアッセイの間隔は、輸血レシピエントに対して重大な危険性を生じ得る。この問題を克服するために、直接ウィルスＲＮＡを検出する核酸に基づく試験（ＮＡＴ）、および抗体応答の代わりにウイルス抗原をアッセイするＨＣＶコア抗原試験が開発された。例えば、Ｋａｓｈｉｗａｋｕｍａら、米国特許第５，８７１，９０４号；Ｂｅｌｄら、Ｔｒａｎｓｆｕｓｉｏｎ（２０００）４０：５７５～５７９を参照のこと。

【０００８】

しかし、適切な患者の看護を提供し、そして血液および血液製剤によるか、または密接な個人的接触によるＨＣＶの感染を防ぐための高感度で正確な診断手段および予後診断手段

の必要性が残っている。

【０００９】

（発明の要旨）

本発明は、ＨＣＶセロコンバーション抗体が、代表的に抗コアおよび抗ＮＳ－３（ヘリカーゼ）であるという知見に一部基づいている。従って、本発明は、単一の固体マトリックスを使用してサンプル中に存在するＨＣＶ抗原およびＨＣＶ抗体の両方を検出し得るＨＣＶコア抗原とＮＳ３抗体との組み合わせアッセイを提供する。

【００１０】

従って、１つの実施形態において、本発明は、少なくとも１つのＨＣＶ抗コア抗体およびそれに結合する少なくとも１つの単離されたＨＣＶ　ＮＳ３／４ａエピトープを含むイムノアッセイ固体支持体に関する。この抗体およびＮＳ３／４ａエピトープは、本明細書中に記載される分子のいずれかであり得る。さらに、固体支持体は、図７Ａ～７Ｆに示されるアミノ酸配列を含む多エピトープ融合抗原のような、本明細書中に記載される多エピトープ融合抗原のいずれかを含み得る。

【００１１】

特定の実施形態において、固体支持体は、それに結合する少なくとも２ゆのＨＣＶ抗コア抗体を含む。さらに、抗コア抗体は、モノクローナル抗体であり得る。さらに、ＮＳ３／４ａエピトープは、コンホメーションエピトープ（例えば、図４Ａ～４Ｄに示されるアミノ酸配列を含むコンホメーションＮＳ３／４ａエピトープ）であり得る。

【００１２】

別の実施形態において、本発明は、少なくとも２つのＨＣＶ抗コアモノクローナル抗体およびそれに結合する少なくとも１つのＨＣＶ　ＮＳ３／４ａコンホメーションエピトープ（図４～４Ｄに示されるアミノ酸配列を含む）を含むイムノアッセイ固体支持体に関する。

【００１３】

なおさらなる実施形態において、本発明は、生物学的サンプル中のＨＣＶ感染を検出する方法に関する。本方法は、以下の工程を包含する：（ａ）上記のようなイムノアッセイ固体支持体を提供する工程；（ｂ）ＨＣＶ抗原およびＨＣＶ抗体（生物学的サンプル中に存在する場合）が、それぞれ少なくとも１つの抗コア抗体およびＮＳ３／４ａエピトープと結合することを可能にする条件下で、生物学的サンプルと固体支持体とを組み合わせる工程；（ｃ）工程（ｂ）からの固体支持体に、複合体が形成する条件下で（ｉ）検出可能に標識した第一の抗体（ここでこの検出可能に標識した第一の抗体は、検出可能に標識されたＨＣＶ抗コア抗体であり、ここでこの標識された抗コア抗体は、固体支持体に結合する少なくとも１つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される）；（ｉｉ）ＮＳ３／４ａエピトープと反応性の生物学的サンプル由来のＨＣＶ抗体と反応する抗原；および（ｉｉｉ）検出可能に標識した第二の抗体（ここで、この検出可能に標識された第二の抗体は、（ｉｉ）の抗原と反応性である）を添加する工程；ならびに（ｄ）抗体と抗原（存在する場合）との間に形成された複合体を、生物学的サンプル中のＨＣＶ感染の指標として検出する工程。ＮＳ３／４ａエピトープは、コンホメーションエピトープ（例えば、図４Ａ～４Ｄに示されるＮＳ３／４ａ配列を有するコンホメーションエピトープ）であり得る。

【００１４】

なお別の実施形態において、本発明は、生物学的サンプル中のＨＣＶ感染を検出する方法に関する。この方法は以下の工程を包含する：（ａ）上記のようなイムノアッセイ固体支持体（それに結合する少なくとも２つのＨＣＶ抗コア抗体を有する）を提供する工程；（ｂ）ＨＣＶ抗原およびＨＣＶ抗体（生物学的サンプル中に存在する場合）が、それぞれ少なくとも２つの抗コア抗体およびＮＳ３／４ａエピトープと結合することを可能にする条件下で、生物学的サンプルと固体支持体とを組み合わせる工程；（ｃ）工程（ｂ）からの固体支持体に、複合体が形成する条件下で（ｉ）検出可能に標識した第一の抗体（ここでこの検出可能に標識した第一の抗体は、検出可能に標識されたＨＣＶ抗コア抗体であり、

ここでこの標識された抗コア抗体は、固体支持体に結合する抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される）；（ｉｉ）ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ；および（ｉｉｉ）検出可能に標識した第二の抗体（ここで、この検出可能に標識された第二の抗体は、ｈＳＯＤアミノ酸配列と反応性であある）を添加する工程；ならびに（ｄ）抗体と抗原（存在する場合）との間に形成された複合体を、生物学的サンプル中のＨＣＶ感染の指標として検出する工程。ＮＳ３／４ａエピトープは、コンホメーションエピトープ（例えば、図４Ａ～４Ｄに示されるＮＳ３／４ａ配列を有するコンホメーションエピトープ）であり得る。

【００１５】

上記実施形態のいずれかにおいて、抗コア抗体は、ＨＣＶコア抗原のＮ末端領域に対して（例えば、ＨＣＶ１ポリタンパク質配列に対して番号付けされた、ＨＣＶのアミノ酸１０－５３に対して）指向され得、そして／または検出可能に標識されたＨＣＶ抗コア抗体は、ＨＣＶコア抗原のＣ末端領域（例えば、ＨＣＶ１ポリタンパク質配列に対して番号付けされたＨＣＶのアミノ酸１２０－１３０）に対して指向され得る。さらに、生物学的サンプルからのＨＣＶ抗体と反応する抗原は、ＮＳ３領域（例えば、ＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ）由来であり得、ヒトスーパーオキシドジスムターゼ（ｈＳＯＤ）アミノ酸配列と融合され得る。この実施形態において、検出可能に標識された第二の抗体は、ｈＳＯＤアミノ酸配列と反応性である。

【００１６】

別の実施形態において、本発明は、生物学的サンプル中のＨＣＶ感染を検出する方法に関する。この方法は以下の工程を包含する：（ａ）２つのＨＣＶ抗コアモノクローナル抗体および図４Ａ～４Ｄに示されるアミノ酸配列を含むコンホメーションエピトープを含むイムノアッセイ固体支持体を提供する工程；（ｂ）ＨＣＶ抗原およびＨＣＶ抗体（生物学的サンプル中に存在する場合）が、それぞれ少なくとも２つの抗コア抗体およびＮＳ３／４ａコンホメーションエピトープと結合することを可能にする条件下で、生物学的サンプルと固体支持体とを組み合わせる工程；（ｃ）工程（ｂ）からの固体支持体に、複合体が形成する条件下で（ｉ）検出可能に標識した第一の抗体（ここでこの検出可能に標識した第一の抗体は、検出可能に標識されたＨＣＶ抗コア抗体であり、ここでこの標識された抗コア抗体は、固体支持体に結合する少なくとも２つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される）；（ｉｉ）ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープ；および（ｉｉｉ）検出可能に標識した第二の抗体（ここで、この検出可能に標識された第二の抗体は、ｈＳＯＤアミノ酸配列と反応性である）を添加する工程；ならびに（ｄ）抗体と抗原（存在する場合）との間に形成された複合体を、生物学的サンプル中のＨＣＶ感染の指標として検出する工程。

【００１７】

特定の実施形態おいて、少なくとも２つの抗コア抗体は、ＨＣＶコア抗原のＮ末端領域に対して（例えば、ＨＣＶ１ポリタンパク質に対して番号付けされた、ＨＣＶのアミノ酸１０－５３に対して）指向され、そして検出可能に標識されたＨＣＶ抗コア抗体は、ＨＣＶコア抗原のＣ末端領域に対して（例えば、ＨＣＶ１ポリタンパク質配列に対して番号付けされたＨＣＶのアミノ酸１２０－１３０に対して）指向される。

【００１８】

別の実施形態において、本発明は、生物学的サンプル中のＨＣＶ感染を検出する方法に関する。この方法は以下の工程を包含する：（ａ）多エピトープ融合抗原を含むイムノアッセイ固体支持体を提供する工程；（ｂ）ＨＣＶ抗原およびＨＣＶ抗体（生物学的サンプル中に存在する場合）が、少なくとも１つの抗コア抗体、ＮＳ３／４ａエピトープおよび多エピトープ融合抗原と結合することを可能にする条件下で、生物学的サンプルと固体支持体とを組み合わせる工程；（ｃ）工程（ｂ）からの固体支持体に、複合体が形成する条件下で（ｉ）検出可能に標識した第一の抗体（ここでこの検出可能に標識した第一の抗体は、検出可能に標識されたＨＣＶ抗コア抗体であり、ここでこの標識された抗コア抗体は、固体支持体に結合する少なくとも１つの抗コア抗体とは異なるＨＣＶコアエピトープに対

して指向される）；（ｉｉ）生物学的サンプル由来のＨＣＶ抗体と反応する第一の抗原および第二の抗原（それぞれ、ＮＳ３／４ａエピトープおよび多エピトープ融合抗原と反応性である）；および（ｉｉｉ）検出可能に標識した第二の抗体（ここで、この検出可能に標識された第二の抗体は、（ｉｉ）の抗原と反応性である）を添加する工程；ならびに（ｄ）抗体と抗原（存在する場合）との間に形成された複合体を、生物学的サンプル中のＨＣＶ感染の指標として検出する工程。

【００１９】

上記のように、抗コア抗体は、ＨＣＶコア抗原のＮ末端領域に対して指向され得、この検出可能に標識された第一のＨＣＶ抗コア抗体は、ＨＣＶコア抗原のＣ末端領域に対して指向され得る。さらに、生物学的サンプル由来のＨＣＶ抗体と反応する第一の抗原は、ＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープを含み得、そしてｈＳＯＤアミノ酸配列と融合され得る。この状況において、検出可能に標識された第二の抗体は、ｈＳＯＤアミノ酸配列と反応性である。さらに、生物学的サンプル由来のＨＣＶ抗体と反応する第二の抗原は、ＨＣＶポリタンパク質のｃ２２領域由来のエピトープ（例えば、ＨＣＶ１ポリタンパク質配列に対して番号付けされた、Ａｒｇ４７の欠失および４４位でのＬｅｕのＴｒｐでの置換を有するＨＣＶポリタンパク質のアミノ酸$Ｌｙｓ_{１０}$～$Ｓｅｒ_{９９}$を含むエピトープ）を含み得る。このエピトープは、ｈＳＯＤアミノ酸配列と融合され得る。その場合、検出可能に標識された第二の抗体は、ｈＳＯＤアミノ酸配列と反応性である。多エピトープ融合抗原は、図７Ａ～７Ｆに示されるアミノ酸配列を含み得る。

【００２０】

なおさらなる実施形態において、本発明は、生物学的サンプルにおけるＨＣＶ感染を検出する方法に関し、この方法は、以下：（ａ）イムノアッセイ固体支持体を提供する工程であって、この固体支持体は、この固体支持体に結合された、２つのＨＣＶ抗コアモノクローナル抗体、ＨＣＶ　ＮＳ３／４ａコンホメーションエピトープ（図４Ａ～４Ｄに記載されるアミノ酸配列を含む）、および多重エピトープ融合抗原（図７Ａ～７Ｆに記載されるアミノ酸配列を含む）を含む、工程；（ｂ）ＨＣＶ抗原および抗体が、生物学的サンプルに存在する場合に、少なくとも２つの抗コア抗体、ＮＳ３／４ａコンホメーションエピトープ、および多重エピトープ融合抗原の各々に結合する条件下で、生物学的サンプルを固体体支持体と合わせる工程；（ｃ）複合体形成条件下で、工程（ｂ）からの固体支持体に、（ｉ）第一の検出可能に標識された抗体（ここで、第一の検出可能に標識された抗体は、検出可能に標識されたＨＣＶ抗コア抗体であり、ここで、この標識された抗コア抗体は、固体支持体に結合された少なくとも２つの抗コア抗体とは異なるＨＣＶコアエピトープに対して指向される）；（ｉｉ）ｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ３３ｃ領域由来のエピトープおよびｈＳＯＤアミノ酸配列に融合されたＨＣＶポリタンパク質のｃ２２領域由来のエピトープ；ならびに（ｉｉｉ）第二の検出可能に標識された抗体（ここで、この第二の検出可能に標識された抗体は、このｈＳＯＤアミノ酸配列と反応性である）を添加する工程；（ｄ）（存在する場合、生物学的サンプルにおけるＨＣＶ感染の指標としての）抗体と抗原との間の複合体を検出する工程、を包含する。

【００２１】

この実施形態において、少なくとも２つの抗コア抗体は、ＨＣＶコア抗原のＮ末端領域に対して（例えば、ＨＣＶのアミノ酸１０～５３（ＨＣＶ１ポリタンパク質に対して番号付けされた）に対して）指向され得、そして検出可能に標識されたＨＣＶ抗コア抗体は、ＨＣＶコア抗原のＣ末端領域に対して（例えば、ＨＣＶのアミノ酸１２０～１３０（ＨＣＶ１ポリタンパク質に対して番号付けされた）に対して）指向される。さらに、ｃ２２領域由来のエピトープは、ＨＣＶ１ポリタンパク質に対して番号付けされた、ＨＣＶポリタンパク質のアミノ酸$Ｌｙｓ_{１０}$～$Ｓｅｒ_{９９}$を含み得、ここで、Ａｒｇ４７が欠失し、そして４４位でＬｕｅがＴｒｐに置換されている。

【００２２】

他の実施形態において、本発明は、免疫診断試験キットに関し、このキットは、上記のイムノアッセイ固体支持体および免疫診断試験を実施するための説明書を含む。

【００２３】
なおさらなる実施形態において、本発明は、イムノアッセイ固体支持体を生成する方法に関し、この方法は、（ａ）固体支持体を提供する工程；および（ｂ）少なくとも１つのＨＣＶ抗コア抗体（例えば、１つまたは２つ以上）、および少なくとも１つの単離されたＨＣＶ　ＮＳ３／４ａエピトープ、ならびに必要に応じて、多重エピトープ融合抗原を、固体支持体に結合する工程を包含する。この抗コア抗体、ＮＳ３／４ａエピトープ、および多重エピトープ融合抗原は、上記の通りである。
【００２４】
さらなる実施形態において、本発明は、多重エピトープ融合抗原に関し、この抗原は、図７Ａ～７Ｆに記載されるアミノ酸配列またはこのアミノ酸配列に対して少なくとも８０％の配列同一性（例えば、９０％以上の配列同一性）を有するアミノ酸配列を含み、このアミノ酸配列は、ＨＣＶ感染個体由来の生物学的サンプル中に存在する抗ＨＣＶ抗体と特異的に反応する。特定の実施形態において、この多重エピトープ融合抗原は、図５Ａ～５Ｆに示されるアミノ酸配列からなる。
【００２５】
さらなる実施形態において、本発明は、上記の多重エピトープ融合抗原についてのコード配列を含むポリヌクレオチド、ポリヌクレオチドを含む組換えベクター、この組換えベクターで形質転換された宿主細胞、ならびに、組換え多重エピトープ融合抗原を作製する方法に関し、この方法は、（ａ）上記のような宿主細胞の集団を提供する工程；および（ｂ）この組換えベクター中に存在するコード配列によってコードされる多重エピトープ融合抗原が発現される条件下でこの細胞の集団を培養する工程を包含する。
【００２６】
本発明のこれらの局面および他の局面は、以下の詳細な説明および添付の図面を参照して、明らかとなる。
【００２７】
（発明の詳細な説明）
本発明の実施は、他に示されない限り、当該分野の技術の範囲内の、化学、生化学、組換えＤＮＡ技術および免疫学の従来の方法を使用する。このような技術は、文献に完全に説明される。例えば、Ｆｕｎｄａｍｅｎｔａｌ　Ｖｉｒｏｌｏｇｙ、第２版、Ｉ巻およびＩＩ巻（Ｂ．Ｎ．ＦｉｅｌｄｓおよびＤ．Ｍ．Ｋｎｉｐｅ，編）；Ｈａｎｄｂｏｏｋ　ｏｆ　Ｅｘｐｅｒｉｍｅｎｔａｌ　Ｉｍｍｕｎｏｌｏｇｙ、Ｉ－ＩＶ巻（Ｄ．Ｍ．ＷｅｉｒおよびＣ．Ｃ．Ｂｌａｃｋｗｅｌｌ編、Ｂｌａｃｋｗｅｌｌ　Ｓｃｉｅｎｔｉｆｉｃ　Ｐｕｂｌｉｃａｔｉｏｎｓ）；Ｔ．Ｅ．Ｃｒｅｉｇｈｔｏｎ、Ｐｒｏｔｅｉｎｓ：Ｓｔｒｕｃｔｕｒｅｓ　ａｎｄ　Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｐｅｒｔｉｅｓ（Ｗ．Ｈ．Ｆｒｅｅｍａｎ　ａｎｄ　Ｃｏｍｐａｎｙ、１９９３）；Ａ．Ｌ．Ｌｅｈｎｉｎｇｅｒ、Ｂｉｏｃｈｅｍｉｓｔｒｙ（Ｗｏｒｔｈ　Ｐｕｂｌｉｓｈｅｒｓ，Ｉｎｃ．、最新添付（ｃｕｒｒｅｎｔ　ａｄｄｉｔｉｏｎ））；Ｓａｍｂｒｏｏｋら，Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ（第２版、１９８９）；Ｍｅｔｈｏｄｓ　Ｉｎ　Ｅｎｚｙｍｏｌｏｇｙ（Ｓ．ＣｏｌｏｗｉｃｋおよびＮ．Ｋａｐｌａｎ編、Ａｃａｄｅｍｉｃ　Ｐｒｅｓｓ，Ｉｎｃ．）。
【００２８】
本明細書および添付の特許請求の範囲で使用される場合、単数形「ａ」、「ａｎ」および「ｔｈｅ」は、内容がそうではないと明らかに示さない限り、複数の参照をも含むことに注意しなければならない。従って、例えば、「抗原」に対する参照は、２以上の抗原の混合物を含む、など。
【００２９】
以下のアミノ酸の略語は、この明細書を通して使用される：

アラニン：Ａｌａ（Ａ）	アルギニン：Ａｒｇ（Ｒ）
アスパラギン：Ａｓｎ（Ｎ）	アスパラギン酸：Ａｓｐ（Ｄ）
システイン：Ｃｙｓ（Ｃ）	グルタミン：Ｇｌｎ（Ｑ）

グルタミン酸：Ｇｌｕ（Ｅ）　　グリシン：Ｇｌｙ（Ｇ）
ヒスチジン：Ｈｉｓ（Ｈ）　　イソロイシン：Ｉｌｅ（Ｉ）
ロイシン：Ｌｅｕ（Ｌ）　　リジン：Ｌｙｓ（Ｋ）
メチオニン：Ｍｅｔ（Ｍ）　　フェニルアラニン：Ｐｈｅ（Ｆ）
プロリン：Ｐｒｏ（Ｐ）　　セリン：Ｓｅｒ（Ｓ）
スレオニン：Ｔｈｒ（Ｔ）　　トリプトファン：Ｔｒｐ（Ｗ）
チロシン：Ｔｙｒ（Ｙ）　　バリン：Ｖａｌ（Ｖ）

（Ｉ．定義）

本発明を記載する際に、以下の用語が使用され、そして以下に示されるように定義されることが意図される。

【００３０】

用語「ポリペプチド」および「タンパク質」は、アミノ酸残基のポリマーをいい。そして生成物の最小長に限定されない。従って、ペプチド、オリゴペプチド、ダイマー、マルチマーなどが、この定義に含まれる。全長タンパク質およびそのフラグメントの両方が、この定義に含まれる。この用語はまた、ポリペプチドの発現後修飾（例えば、グルコシル化、アセチル化、リン酸化など）を含む。さらに、本発明の目的のために、「ポリペプチド」は、タンパク質の所望の活性が維持される限り、ネイティブな配列に対する、改変（例えば、欠失、付加および置換（一般に、性質が保存的である））を含むタンパク質をいう。これらの改変は、部位特異的変異誘発を介してのように意図的であり得るか、またはこのタンパク質を産生する宿主の変異もしくはＰＣＲ増幅に起因するエラーを介するような、偶発的なものであり得る。

【００３１】

ＨＣＶポリペプチドは、上記のように、ＨＣＶポリタンパク質に由来するポリペプチドである。このポリペプチドは、物理的にＨＣＶに由来する必要はなく、合成的にまたは組換え的に生成され得る。さらに、このポリペプチドは、種々のＨＣＶ株および単離物のいずれか（例えば、限定されないが、ＨＣＶの株１、２、３、または４由来の単離物のいずれか）に由来し得る。多くの保存領域および可変領域が、これらの株で既知であり、そして一般に、これらの領域に由来するエピトープのアミノ酸配列は、２つの配列が整列された場合に、高い程度の配列相同性（例えば、３０％より大きい、好ましくは４０％より大きいアミノ酸配列相同性）を有する。従って、例えば、用語「ＮＳ３／４ａ」ポリペプチドは、種々のＨＣＶ株のいずれかに由来するネイティブなＮＳ３／４ａ、ならびに以下にさらに定義されるようなＮＳ３／４ａアナログ、ムテインおよび免疫原性フラグメントをいう。多くのこれらの株の完全な遺伝子型は、公知である。例えば、米国特許第６，１５０，０８７号およびＧｅｎＢａｎｋ登録番号ＡＪ２３８８００ならびにＡＪ２３８７９９を参照のこと。

【００３２】

用語「アナログ」および「ムテイン」は、参照分子の生物学的に活性な誘導体またはこのような誘導体のフラグメント（これらは、本明細書中に記載されるアッセイにおいて免疫反応性のような所望の活性を維持する）をいう。一般に、用語「アナログ」は、改変が免疫原性活性を破壊しない限り、ネイティブ分子に対して、１以上のアミノ酸付加、置換（一般に、性質が保存的である）、および／または欠失を有するネイティブなポリペプチド配列および構造を有する化合物をいう。用語「ムテイン」は、１つ以上のペプチド模倣物（「ペプトイド」）を有するペプチドをいい、例えば、国際公開番号ＷＯ　９１／０４２８２に記載される。好ましくは、このアナログまたはムテインは、ネイティブな分子と少なくとも同じ免疫活性を有する。ポリペプチドアナログおよびムテインを作製するための方法は、当該分野で公知であり、そして以下にさらに記載される。

【００３３】

特に好ましいアナログは、性質が保存的である置換（すなわち、それらの側鎖が関係するアミノ酸のファミリー内で起こる置換）を含む。具体的に、アミノ酸は、一般的に４つのファミリーに分類される：（１）酸性－－アスパラギン酸およびグルタミン酸；（２）塩

基性－－リジン、アルギニン、ヒスチジン；（３）非極性－－アラニン、バリン、ロイシン、イソロイシン、プロリン、フェニルアラニン、メチオニン、トリプトファン；および（４）無電荷で極性－－グリシン、アスパラギン、グルタミン、システイン、セリン、スレオニン、チロシン。フェニルアラニン、トリプトファン、およびチロシンは、しばしば、芳香族アミノ酸として分類される。例えば、ロイシンのイソロイシンまたはバリンでの、アスパラギン酸のグルタミン酸での、スレオニンのセリンでの単独置換、またはアミノ酸の構造的に関連したアミノ酸の類似の保存的な置換は、生物学的活性に対して、主要な効果は有さないことが、合理的に予測可能である。例えば、目的のポリペプチドは、分子の所望の機能がインタクトなままである限り、約５～１０までの保存的アミノ酸置換または非保存的アミノ酸置換、もしくは約１５～２５までの保存的アミノ酸置換または非保存的アミノ酸置換、あるいは５～２５の間の任意の整数の保存的アミノ酸置換または非保存的アミノ酸置換を有し得る。当業者は、当該分野で周知の、Ｈｏｐｐ／ＷｏｏｄｓおよびＫｙｔｅ－Ｄｏｏｌｉｔｔｌｅプロットを参照することによって、変化を許容し得る目的の分子の領域を容易に決定し得る。

【００３４】

「フラグメント」は、インタクトな全長ポリペプチド配列および構造の一部のみからなるポリペプチドを意図する。このフラグメントは、ネイティブなポリペプチドのＣ末端欠失および／またはＮ末端欠失を含み得る。特定のＨＣＶタンパク質の「免疫原性フラグメント」は、一般に、全長分子の少なくとも約５～１０連続するアミノ酸残基、好ましくは全長分子の少なくとも約１５～２５連続するアミノ酸残基、および最も好ましくは全長分子の少なくとも約２０～５０以上連続するアミノ酸残基（これらは、エピトープを規定する）を含むか、または５個のアミノ酸と全長配列との間の任意の整数の連続するアミノ酸残基を含むが、ただし、問題のフラグメントは、本明細書中に記載されるアッセイにおいて免疫反応性を保持する。例えば、好ましい免疫原性フラグメントとしては、例えば、ポリタンパク質のアミノ酸１０～４５、１０～５３、６７～８８、および１２０～１３０、エピトープ５－１－１（ウイルスゲノムのＮＳ３領域において）ならびにＨＣＶポリタンパク質のＥ１、Ｅ２、ｃ３３ｃ（ＮＳ３）、ｃ１００（ＮＳ４）、ＮＳ３／４ａおよびＮＳ５領域由来の規定されたエピトープ、ならびにＨＣＶポリタンパク質から同定されるタンパク質の種々のエピトープのいずれかを含むＨＣＶコアのフラグメントが挙げられるが、これらに限定されない。例えば、Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９２）８９：１００１１－１００１５；Ｃｈｉｅｎら、Ｊ．Ｇａｓｔｒｏｅｎｔ．Ｈｅｐａｔｏｌ．（１９９３）８：Ｓ３３－３９；Ｃｈｉｅｎら、国際公開番号ＷＯ　９３／００３６５；Ｃｈｉｅｎ、Ｄ．Ｙ．、国際公開番号ＷＯ　９４／０１７７８；米国特許第６，１５０，０８７号および同第６，１２１，０２０号を参照のこと。

【００３５】

本明細書中で使用される場合、用語「エピトープ」は、少なくとも約３～５、好ましくは約５～１０または１５、および約１０００アミノ酸以下（またはこの間の任意の整数のアミノ酸）の配列をいい、これは、それ自体によって、またはより大きな配列の一部として、このような配列に応答して産生される抗体に結合する配列を規定する。フラグメントの長さに臨界的な上限は存在せず、このフラグメントは、ほぼ全長のタンパク質配列、またはＨＣＶポリタンパク質由来の２つ以上のエピトープを含む融合タンパク質でさえ含み得る。本発明における使用のためのエピトープは、これが由来する親タンパク質の一部分の正確な配列を有するポリペプチドに限定されない。実際に、ウイルスゲノムは、一定のフラックスの状態にあり、そして単離物間で比較的高い程度の変異性を示すいくつかの可変ドメインを含む。従って、用語「エピトープ」は、ネイティブな配列に同一な配列、ならびにネイティブな配列に対する改変（例えば、欠失、付加および置換（一般に、性質が保存的である））を包含する。

【００３６】

エピトープを含む所定のポリペプチドの領域は、当該分野で周知の、任意の数のエピトープマッピング技術を使用して、同定され得る。例えば、Ｅｐｉｔｏｐｅ　Ｍａｐｐｉｎｇ

Ｐｒｏｔｏｃｏｌｓ，Ｍｅｔｈｏｄｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ、第６６巻（Ｇｌｅｎｎ Ｅ．Ｍｏｒｒｉｓ編、１９９６）Ｈｕｍａｎａ Ｐｒｅｓｓ、Ｔｏｔｏｗａ、Ｎｅｗ Ｊｅｒｓｅｙを参照のこと。例えば、線状エピトープは、例えば、多数のペプチド（これらのペプチドは、タンパク質分子の一部分に対応する）を固体支持体上で同時に合成し、そしてこれらのペプチドを固体支持体になおも付着させつつ、これらのペプチドを抗体を反応させることによって決定され得る。このような技術は、当該分野で公知であり、そして例えば以下に記載される：米国特許第４，７０８，８７１号；Ｇｅｙｓｅｎら（１９８４）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８１：３９９８－４００２；Ｇｅｙｓｅｎら（１９８５）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８２：１７８－１８２；Ｇｅｙｓｅｎら（１９８６）Ｍｏｌｅｃ．Ｉｍｍｕｎｏｌ．２３：７０９－７１５。このような技術を使用して、ＨＣＶの多くのエピトープが同定されている。例えば、Ｃｈｉｅｎら、Ｖｉｒａｌ Ｈｅｐａｔｉｔｉｓ ａｎｄ Ｌｉｖｅｒ Ｄｉｓｅａｓｅ（１９９４）３２０－３２４頁、およびさらに以下を参照のこと。同様に、コンホメーションエピトープは、例えば、Ｘ線結晶学および２次元核磁気共鳴によって、アミノ酸の空間的立体配座を決定することによって容易に同定される。例えば、Ｅｐｉｔｏｐｅ Ｍａｐｐｉｎｇ Ｐｒｏｔｏｃｏｌｓ（前出）を参照のこと。タンパク質の抗原性領域はまた、標準的な抗原性および疎水性親水性指標プロット（例えば、Ｏｘｆｏｒｄ Ｍｏｌｅｃｕｌａｒ Ｇｒｏｕｐから入手可能なＯｍｉｇａ ｖｅｒｓｉｏｎ １．０ソフトウェアプログラムを使用して計算されたもの）を使用して、同定され得る。このコンピュータプログラムは、抗原性プロフィールを決定するためのＨｏｐｐ／Ｗｏｏｄｓ法（Ｈｏｐｐら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ ＵＳＡ（１９８１）７８：３８２４－３８２８）、および疎水性親水性指標プロットのためのＫｙｔｅ－Ｄｏｏｌｉｔｔｌｅ技術（Ｋｙｔｅら、Ｊ．Ｍｏｌ．Ｂｉｏｌ．（１９８２）１５７：１０５－１３２）を使用する。

【００３７】

本明細書中で使用される場合、用語「コンホメーションエピトープ」とは、全長の天然のタンパク質内の、エピトープをコードするアミノ酸配列にネイティブな構造的特徴を有する、全長タンパク質、またはそのアナログまたはムテインの一部をいう。ネイティブな構造的特徴としては、グリコシル化および三次元構造が挙げられるが、これらに限定されない。エピトープ規定配列の長さは、幅広い変動に供され得る。なぜなら、これらのエピトープは、抗原の三次元形状（例えば、折り畳み）によって形成されると考えられるからである。従って、エピトープを規定するアミノ酸は、比較的少ない数であり得るが、分子の長さに沿って（またはダイマーなどの場合には、異なる分子上にさえ）幅広く分散し得、折り畳みにより正確なエピトープコンホメーションにされる。エピトープを規定する残基間の抗原の部分は、エピトープのコンホメーショナルな構造に重要ではなくてもよい。例えば、これらの介在する配列の欠失または置換は、エピトープのコンホメーションに重要な配列（例えば、ジスルフィド結合に関与するシステイン、グリコシル化部位など）が維持される限り、コンホメーションエピトープに影響しなくてもよい。

【００３８】

ＮＳ３／４ａ領域に存在するコンホメーションエピトープは、上で議論される方法を使用して容易に同定される。さらに、所与のポリペプチドにおけるコンホメーションエピトープの存在または非存在は、抗体（コンホメーションエピトープに対するポリクローナル血清またはモノクローナル血清）を用いて目的の抗原をスクリーニングし、そしてその反応性を線形のエピトープのみ（存在する場合）を保持する変性版の抗原の反応性と比較することによって容易に決定され得る。ポリクローナル抗体を使用するこのようなスクリーニングにおいて、ポリクローナル血清を、最初に変性抗原を用いて吸収し、そして目的の抗原に対する抗体を保持するか否かを観測することが有利であり得る。さらに、ＮＳ３／４ａの場合において、ネイティブなコンホメーションを保存する分子はまた、プロテアーゼ酵素活性そして必要に応じて、ヘリカーゼ酵素活性を有する。このような活性は、以下にさらに記載されるように、酵素アッセイを使用して、検出され得る。

【００３９】
好ましくは、コンホメーションエピトープは、組換え的に産生され、そして、例えば、エピトープの変性なしにその所望の構造的特徴を保存する条件下で抽出可能である細胞において発現される。このような細胞としては、細菌細胞、酵母細胞、昆虫細胞、および哺乳動物細胞が挙げられる。ＨＣＶポリタンパク質からの組換えコンホメーションエピトープの発現および単離は、例えば、国際公開番号ＷＯ９６／０４３０１、ＷＯ９４／０１７７８、ＷＯ９５／３３０５３、ＷＯ９２／０８７３４に記載される。あるいは、抗原を発現し、そしてさらに、回収の後にタンパク質を再生することが可能である。化学合成によってまた、「ネイティブ」な抗原のコンホメーションエピトープと交差反応するコンホメーショナル抗原ミミトープ（ｍｉｍｉｔｏｐｅ）が提供され得ることがまた理解される。

【００４０】
本明細書中で使用される場合、用語「多エピトープ融合抗原」または「ＭＥＦＡ」は、複数のＨＣＶ抗原がアミノ酸の単一の連続した鎖の一部であるポリペプチドを意図し、この鎖は、天然に存在しない。ＨＣＶ抗原は、ペプチド結合によって互いに直接的に結合され得るか、または介在するアミノ酸配列によって分離され得る。融合抗原はまた、ＨＣＶポリタンパク質に対して外来性の配列を含み得る。さらに、存在するＨＣＶ配列は、複数のゲノム型由来であり得、そして／またはＨＣＶの単離体であり得る。本発明のイムノアッセイにおける使用のための特定のＭＥＦＡの例は、例えば、国際公開番号ＷＯ９７／４４４６９に詳細に記載され、そして以下にさらに記載される。

【００４１】
「抗体」は、化学的手段または物理的手段によって、目的のポリペプチドに特異的に結合する分子を意図する。従って、ＨＣＶコア抗体は、ＨＣＶコアタンパク質に特異的に結合する分子である。本明細書中で使用される場合、用語「抗体」は、ポリクローナル調製物およびモノクローナル調製物の両方、ならびに以下：ハイブリッド（キメラ）抗体分子（例えば、Ｗｉｎｔｅｒら（１９９１）Ｎａｔｕｒｅ ３４９：２９３～２９９；および米国特許番号第４，８１６，５６７号を参照のこと）；Ｆ（ａｂ’）$_2$フラグメントおよびＦ（ａｂ）フラグメント；Ｆｖ分子（非共有結合ヘテロダイマー、例えば、Ｉｎｂａｒら（１９７２）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ６９：２６５９～２６６２；およびＥｈｒｌｉｃｈら（１９８０）Ｂｉｏｃｈｅｍ １９：４０９１～４０９６を参照のこと）；単鎖Ｆｖ分子（ｓＦｖ）（例えば、Ｈｕｓｔｏｎら（１９８８）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ８５：５８７９～５８８３を参照のこと）；二量体および三量体の抗体フラグメント構築物；ミニ体（ｍｉｎｉｂｏｄｉｅｓ）（例えば、Ｐａｃｋら（１９９２）Ｂｉｏｃｈｅｍ ３１：１５７９～１５８４；Ｃｕｍｂｅｒら（１９９２）Ｊ Ｉｍｍｕｎｏｌｏｇｙ １４９Ｂ：１２０～１２６を参照のこと）；ヒト化抗体分子（例えば、Ｒｉｅｃｈｍａｎｎら（１９８８）Ｎａｔｕｒｅ ３３２：３２３～３２７；Ｖｅｒｈｏｅｙａｎら（１９８８）Ｓｃｉｅｎｃｅ ２３９：１５３４～１５３６；および１９９４年９月２１日に公開された英国特許公開番号ＧＢ２，２７６，１６９を参照のこと）；ならびに、このような分子から得られる任意の機能性フラグメントから得られる抗体を含み、ここで、このようなフラグメントは、親の抗体分子の免疫学的結合特性を保持する。

【００４２】
本明細書中で使用される場合、用語「モノクローナル抗体」とは、均一な抗体集団を有する抗体組成物をいう。この用語は、抗体の種または供給源に関して限定されず、それが作製される方法によっても限定されることを意図しない。従って、この用語は、マウスハイブリドーマから得た抗体、ならびにマウスハイブリドーマよりもむしろヒトを使用して得たヒトモノクローナル抗体を包含する。例えば、Ｃｏｔｅら、Ｍｏｎｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ ａｎｄ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ，Ａｌａｎ Ｒ．Ｌｉｓｓ，１９８５，ｐ．７７を参照のこと。

【００４３】
「組換え体」タンパク質は、所望の活性を有し、そして本明細書中に記載される組換えＤ

ＮＡ技術によって調製されたタンパク質である。一般的に、目的の遺伝子は、以下にさらに記載のように、クローン化され、次いで、形質転換された生物において発現される。宿主生物は、発現条件下で外来遺伝子を発現してタンパク質を産生する。

【００４４】

「単離された」とは、ポリペプチドをいう場合、示された分子が、その分子が天然に見出される生物全体から分離され、そして別々であるか、または同じ型の他の高分子の実質的な非存在下で存在することを意味する。ポリヌクレオチドに関して、用語「単離された」は、天然においてそのポリヌクレオチドと通常関連する配列の全てまたは一部を欠いた核酸分子；または天然に存在するが、その配列に関連する異種配列を有する配列；または染色体から解離した分子である。

【００４５】

「等価な抗原決定基」とは、ＨＣＶの異なる亜種または株（例えば、ＨＣＶの株１、２または３）由来の抗原決定基を意味する。より詳細には、エピトープ（例えば、５－１－１）が、公知であり、そしてこのようなエピトープは、株１、２および３の間で変わる。従って、３つの異なる株由来のエピトープ５－１－１は、等価な抗原決定基であり、従って、たとえそれらの配列が同一でないとしても、「コピー」である。一般的に、等価な抗原決定基のアミノ酸配列は、２つの配列が整列された場合、高い程度の配列相同性（例えば、３０％より大きい、好ましくは４０％より大きい、アミノ酸配列相同性）を有する。

【００４６】

「相同性」とは、２つのポリヌクレオチド部分または２つのポリペプチド部分の間の類似性の割合をいう。２つのＤＮＡ配列、または２つのポリペプチド配列は、これらの配列が、規定された長さの分子にわたって、少なくとも約５０％、好ましくは少なくとも約７５％、より好ましくは少なくとも約８０％～８５％、好ましくは少なくとも約９０％、そして最も好ましくは少なくとも約９５％～９８％の配列類似性を示す場合、互いに「実質的に相同」である。本明細書中で使用される場合、実質的に相同はまた、特定のＤＮＡ配列またはポリペプチド配列に対して完全な同一性を示す配列をいう。

【００４７】

一般的に、「同一性」とは、２つのポリヌクレオチド配列またはポリペプチド配列それぞれの正確なヌクレオチド－ヌクレオチドの対応またはアミノ酸－アミノ酸の対応をいう。同一性の割合は、それらの配列を整列させ、２つの整列された配列の間の一致の正確な数を数え、短い方の配列の長さによって割り、そして結果に１００を掛けることによる、２つの分子間の配列情報の直接的な比較によって決定され得る。

【００４８】

容易に入手可能なコンピュータプログラム（例えば、ＡＬＩＧＮ，Ｄａｙｈｏｆｆ，Ｍ．Ｏ．、Ａｔｌａｓ ｏｆ Ｐｒｏｔｅｉｎ Ｓｅｑｕｅｎｃｅ ａｎｄ Ｓｔｒｕｃｔｕｒｅ Ｍ．Ｏ．Ｄａｙｈｏｆｆ編、補遺５、３：３５３～３５８，Ｎａｔｉｏｎａｌ ｂｉｏｍｅｄｉｃａｌ Ｒｅｓｅａｒｃｈ Ｆｏｕｎｄａｔｉｏｎ，Ｗａｓｈｉｎｇｔｏｎ，ＤＣ（これは、ペプチド分析のために、ＳｍｉｔｈおよびＷａｔｅｒｍａｎ Ａｄｖａｎｃｅｓ ｉｎ Ａｐｐｌ．Ｍａｔｈ．２：４８２～４８９，１９８１の局所的相同性アルゴリズムを適合させる））は、類似性または同一性の分析を助けるために使用され得る。ヌクレオチド配列類似性および同一性を決定するためのプログラム（例えば、ＢＥＳＴＦＩＴ、ＦＡＳＴＡおよびＧＡＰプログラム（これらもまた、ＳｍｉｔｈおよびＷａｔｅｒｍａｎのアルゴリズムに依存する））は、Ｗｉｓｃｏｎｓｉｎ Ｓｅｑｕｅｎｃｅ Ａｎａｌｙｓｉｓ Ｐａｃｋａｇｅ，Ｖｅｒｓｉｏｎ ８（Ｇｅｎｅｔｉｃｓ Ｃｏｍｐｕｔｅｒ Ｇｒｏｕｐ，Ｍａｄｉｓｏｎ，ＷＩから入手可能）において入手可能である。これらのプログラムは、製造業者によって推奨され、そして上に参照されるＷｉｓｃｏｎｓｉｎ Ｓｅｑｕｅｎｃｅ Ａｎａｌｙｓｉｓ Ｐａｃｋａｇｅに記載されるデフォルトパラメーターを用いて容易に使用される。例えば、参照配列に対する特定のヌクレオチド配列の相同性の割合は、６つのヌクレオチド位置のデフォルトスコアリングテーブルおよびギャップペナルティーを用いてＳｍｉｔｈおよびＷａｔｅｒｍａｎの相同性アルゴリズム

を使用して決定され得る。

【００４９】

本発明の状況において類似性の割合を確立する別の方法は、Ｕｎｉｖｅｒｓｉｔｙ ｏｆ Ｅｄｉｎｂｕｒｇｈによる著作権があり、Ｊｏｈｎ Ｆ．ＣｏｌｌｉｎｓおよびＳｈａｎｅ Ｓ．Ｓｔｕｒｒｏｋによって開発され、そしてＩｎｔｅｌｌｉＧｅｎｅｔｉｃｓ，Ｉｎｃ．（Ｍｏｕｎｔａｉｎ Ｖｉｅｗ，ＣＡ）によって配給されるプログラムのＭＰＳＲＣＨパッケージを使用することである。この一そろいのパッケージから、Ｓｍｉｔｈ－Ｗａｔｅｒｍａｎアルゴリズムを使用し得、ここで、デフォルトパラメーターは、スコアリングテーブルのために使用される（例えば、１２のギャップオープンペナルティー、１のギャップ伸長ペナルティー、および６のギャップ）。生成されるデータから、「一致」値が「配列類似性」を反映する。配列間の同一性の割合または類似性の割合を計算するための他の適切なプログラムは、当該分野において一般的に公知であり、例えば、別の整列プログラムは、デフォルトパラメーターとともに使用されるＢＬＡＳＴである。例えば、ＢＬＡＳＴＮおよびＢＬＡＳＴＰは、以下のデフォルトパラメーターを使用して用いられ得る：遺伝子コード＝標準；フィルター＝なし；鎖＝両方；カットオフ＝６０；期待値＝１０；マトリクス（Ｍａｔｒｉｘ）＝ＢＬＯＳＵＭ６２；記載（Ｄｅｓｃｒｉｐｔｉｏｎｓ）＝５０配列；ソート＝ＨＩＧＨ ＳＣＯＲＥ；データベース（Ｄａｔａｂａｓｅ）＝縮重なし、ＧｅｎＢａｎｋ＋ＥＭＢＬ＋ＤＤＢＪ＋ＰＤＢ＋ＧｅｎＢａｎｋ ＣＤＳ ｔｒａｎｓｌａｔｉｏｎｓ＋Ｓｗｉｓｓ ｐｒｏｔｅｉｎ＋Ｓｐｕｐｄａｔｅ＋ＰＩＲ。これらのプログラムの詳細は、以下のインターネットアドレスにおいて見出され得る：ｈｔｔｐ：／／ｗｗｗ．ｎｃｂｉ．ｎｌｍ．ｇｏｖ／ｃｇｉ－ｂｉｎ／ＢＬＡＳＴ。

【００５０】

あるいは、相同性は、相同な領域間に安定な二重鎖を形成する条件下でのポリペプチドのハイブリダイゼーション、続いて、単鎖特異的ヌクレアーゼでの消化、および消化されたフラグメントのサイズの決定によって決定され得る。実質的に相同であるＤＮＡ配列は、その特定の系について規定されるように、例えばストリンジェントな条件下で、サザンハイブリダイゼーション実験で同定され得る。適切なハイブリダイゼーション条件を規定することは、当該分野の技術の範囲内である。例えば、Ｓａｍｂｒｏｏｋら、上記；ＤＮＡ Ｃｌｏｎｉｎｇ上記；Ｎｕｃｌｅｉｃ Ａｃｉｄ Ｈｙｂｒｉｄｉｚａｔｉｏｎ、上記を参照のこと。

【００５１】

「コード配列」または選択されたポリペプチドを「コード」する配列は、適切な調節配列の制御下に配置される場合、インビトロまたはインビボで、転写され（ＤＮＡの場合）、ポリペプチドに翻訳される（ｍＲＮＡの場合）核酸分子である。コード配列の境界は、５’（アミノ）末端における開始コドンおよび３’（カルボキシ）末端における翻訳終止コドンによって決定される。転写終結配列は、コード配列の３’側に配置され得る。

【００５２】

「作動可能に連結される」とは、そのように記載される成分が、それらの所望の機能を実行するように構成されるエレメントの配置をいう。従って、コード配列に作動可能に連結される所与のプロモーターは、適切な転写因子などが存在する場合、コード配列の発現をもたらし得る。プロモーターは、コード配列の発現を指示するように機能する限り、コード配列と連続である必要はない。従って、例えば、介在する、翻訳されないが転写される配列が、プロモーター配列とコード配列との間に存在し得（イントロンが転写され得るように）、プロモーター配列は、なお、コード配列に「作動可能に連結される」とみなされ得る。

【００５３】

「制御エレメント」とは、それが連結するコード配列の発現を助けるポリヌクレオチド配列をいう。この用語は、プロモーター、転写終結配列、上流調節ドメイン、ポリアデニル化シグナル、非翻訳領域（５’－ＵＴＲおよび３’－ＵＴＲを含む）、ならびに適切な場合、リーダー配列およびエンハンサー（これらは、集合的に、宿主細胞においてコード配

列の転写および翻訳を提供する）を含む。

【００５４】

本明細書中で使用される場合、「プロモーター」は、宿主細胞においてＲＮＡポリメラーゼを結合し得、それに作動可能に連結される下流の（３’方向）コード配列の転写を開始し得るＤＮＡ調節領域である。本発明の目的のために、プロモーター配列は、バックグラウンドよりも上の検出可能なレベルで目的の遺伝子の転写を開始するのに必要な最小数の塩基またはエレメントを含む。プロモーター配列には、転写開始部位、ならびにＲＮＡポリメラーゼの結合を担うタンパク質結合ドメイン（コンセンサス配列）がある。真核生物プロモーターは、しばしば、常にではないが、「ＴＡＴＡ」ボックスおよび「ＣＡＴ」ボックスを含む。

【００５５】

制御配列は、ＲＮＡポリメラーゼがプロモーター配列に結合し、ｍＲＮＡにコード配列を転写する場合、細胞においてコード配列の「転写を指示し」、次いで、このｍＲＮＡは、コード配列によってコードされるポリペプチドに翻訳される。

【００５６】

「発現カセット」または「発現構築物」とは、目的の配列または遺伝子の発現を指向し得るアセンブリをいう。この発現カセットは、上記のような制御エレメント（例えば、目的の配列または遺伝子に（これらの転写を指向するように）作動可能に連結されるプロモーター）を含み、そしてしばしば、同様に、ポリアデニル化配列を含む。本発明の特定の実施形態において、本明細書中に記載される発現カセットは、プラスミド構築物内に含まれ得る。この発現カセットの構成成分に加えて、このプラスミド構築物はまた、１種以上の選択マーカー、このプラスミド構築物が単鎖ＤＮＡとして存在することを可能にするシグナル（例えば、Ｍ１３の複製起点）、少なくとも１ヶ所のマルチクローニングサイト、および「哺乳動物」の複製起点（例えば、ＳＶ４０複製起点またはアデノウイルス複製起点）を含み得る。

【００５７】

本明細書中で使用される「形質転換」とは、挿入のために使用される方法（例えば、直接的な取り込み、トランスフェクション、感染などによる形質転換）に係わらず、外因性ポリヌクレオチドの宿主細胞への挿入をいう。トランスフェクションの特定の方法については、以下をさらに参照のこと。外因性ポリヌクレオチドは、組み込まれていないベクター（例えば、エピソーム）として維持され得るか、または宿主ゲノムに組み込まれ得る。

【００５８】

「宿主細胞」とは、外因性ＤＮＡ配列によって、形質転換された細胞、または形質転換され得る細胞をいう。

【００５９】

「共通の固体支持体」とは、被験体のイムノアッセイにおいて使用されるＨＣＶポリペプチドが、共有結合または疎水性吸着のような非共有結合手段で結合している、単一の固体基質をいう。

【００６０】

「免疫学的反応」とは、目的の抗原が、ＨＣＶに感染した個体由来の生物学的サンプル中に存在する抗ＨＣＶ抗体と特異的に反応することを意味する。

【００６１】

「免疫複合体」とは、抗体が抗原上のエピトープに結合する場合に形成される組み合わせを意図する。

【００６２】

本明細書中で使用される「生物学的サンプル」とは、被験体から単離された組織または流体のサンプルをいい、このサンプルとしては、以下が挙げられるが、これらに限定されない：血液、血漿、血清、糞便、尿、骨髄、胆汁、髄液、リンパ液、皮膚のサンプル、以下の外部分泌物（皮膚、気道、腸管、および尿生殖路）、涙、唾液、乳汁、血球、器官、生検材料、ならびにまた培養培地（例えば、組換え細胞、および細胞成分）中での細胞およ

び組織の増殖から得られる馴化培地を含むがこれに限定されないインビトロ細胞培養構築物のサンプル。

【００６３】

本明細書中で使用される場合、用語「標識」および「検出可能な標識」とは、以下を含むが、これらに限定されない検出可能な分子をいう：放射活性同位元素、蛍光剤、化学ルミネッセンサー（ｃｈｅｍｉｌｕｍｉｎｅｓｃｅｒ）、発光団、酵素、酵素基質、酵素補因子、酵素インヒビター、発光団、色素、金属イオン、金属ゾル、リガンド（例えば、ビオチン、ストレプトアビジン（ｓｔｒｅｐａｖｉｄｉｎｅ）またはハプテン）など。用語「蛍光剤」とは、検出可能な範囲で、蛍光を示し得る物質またはその一部をいう。本発明において使用され得る標識の特定の例としては、以下が挙げられるが、これらに限定されない：西洋ワサビペルオキシダーゼ（ＨＲＰ）、フルオレセイン、ＦＩＴＣ、ローダミン、ダンシル、ウンベリフェロン、ジメチルアクリジニウムエステル（ＤＭＡＥ）、Ｔｅｘａｓレッド、ルミノール、ＮＡＤＰＨおよびα－β－ガラクトシラーゼ。

【００６４】

（ＩＩ．発明の実施の形態）

本発明を詳細に記載する前に、本発明は、特定の式に限定されず、プロセスパラメータ自体は、当然、変化し得ることが理解されなければならない。本明細書中で使用される技術が、本発明の特定の実施形態を記載する目的のみのためであり、限定することを意図しないことも理解されなければならない。

【００６５】

本明細書中に記載される組成物および方法と類似しているかまたは等価の、多数の組成物および方法が、本発明の実施に使用され得るが、好ましい材料および方法は、本明細書中に記載されている。

【００６６】

上記のように、本発明は、初期ＨＣＶ感染を正確に検出するための、新規の診断方法の発見に基づく。この方法は、高度に免疫原性のＨＣＶ抗体およびＨＣＶ抗原の同定および使用に依存し、このＨＣＶ抗体および抗原は、ＨＣＶのセロコンバージョンの初期段階中に存在し、これによって、検出精度を上昇させ、そして誤った結果の発生を減少させる。この方法は、単一のアッセイフォーマットにおいて都合良く実施され得る。

【００６７】

より具体的には、このアッセイは、１種以上のＨＣＶ抗コア抗体（同一のＨＣＶコアエピトープまたは異なるＨＣＶコアエピトープのいずれかに対して指向される）およびＨＣＶポリタンパク質のＮＳ３／４ａ領域由来のエピトープが結合されている固体支持体で実施される。本発明において有用な特定の抗コア抗体の例としては、以下が挙げられるが、これらに限定されない：アミノ酸１０位～５３位の間；アミノ酸１０位～４５位の間；アミノ酸６７位～８８位の間；アミノ酸１２０位～１３０位の間に見出されるコア領域におけるエピトープ対して指向されるモノクローナル抗体のような抗体分子、あるいは例えば、以下において同定されたコアエピトープのいずれかに対して指向される抗体：Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号；Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９２）８９：１００１１－１００１５；Ｃｈｉｅｎら、Ｊ．Ｇａｓｔｒｏｅｎｔ．Ｈｅｐａｔｏｌ．（１９９３）８：Ｓ３３－３９；Ｃｈｉｅｎら、国際公開番号ＷＯ９３／００３６５；Ｃｈｉｅｎ，Ｄ．Ｙ．、国際公開番号ＷＯ９４／０１７７８；ならびに共有に係る、特許になった米国特許出願番号０８／４０３，５９０および同０８／４４４，８１８。

【００６８】

ＨＣＶポリタンパク質のＮＳ３／４ａ領域が記載されており、このアミノ酸配列およびこのタンパク質の全体構造は、例えば、Ｙａｏら、Ｓｔｒｕｃｔｕｒｅ（１９９９年１１月）７：１３５３－１３６３；Ｓａｌｉら、Ｂｉｏｃｈｅｍ．（１９９８）３７：３３９２－３４０１；およびＢａｒｔｅｎｓｃｈｌａｇｅｒ，Ｒ．，Ｊ．Ｖｉｒａｌ Ｈｅｐａｔ．（１９９９）６：１６５－１８１に開示されている。また、Ｄａｓｍａｈａｐａｔｒａ

ら、米国特許第５，８４３，７５２号を参照のこと。本発明のイムノアッセイは、天然に存在するＨＣＶ粒子またはその感染産物において見出されるようなコンホメーション中に存在する、ＮＳ３／４ａ領域由来の少なくとも１種類のコンホメーションエピトープを利用し、プロテアーゼ、必要に応じて、ＮＳ３／４ａ遺伝子産物によって通常示されるヘリカーゼ酵素活性および／またはＨＣＶ感染被験体由来の生物学的サンプル中での抗原と抗体との免疫活性の保持、ならびに抗原の変性におけるエピトープ免疫活性の減少によって明らかとなる。例えば、このコンホメーションエピトープは、加熱、極度に酸性または塩基性のｐＨの変化によって、または公知の有機的変性（例えば、ジチオトレイトール（ＤＴＴ）または適切な界面活性剤）を添加することによって崩壊され得る。例えば、Ｐｒｏｔｅｉｎ Ｐｕｒｉｆｉｃａｔｉｏｎ Ｍｅｔｈｏｄｓ，ａ ｐｒａｃｔｉｃａｌ ａｐｐｒｏａｃｈ（Ｅ．Ｌ．Ｖ．ＨａｒｒｉｓおよびＳ．Ａｎｇａｌ編、ＩＲＬ Ｐｒｅｓｓ）、上記のように処理されない産物と比較される変性産物を参照ここと。

【００６９】

プロテアーゼ活性およびヘリカーゼ活性は、当該分野で周知の標準的な酵素アッセイを使用して決定され得る。例えば、プロテアーゼ活性は、当該分野で周知のアッセイの使用により決定され得る。例えば、Ｔａｋｅｓｈｉｔａら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．（１９９７）２４７：２４２－２４６；Ｋａｋｉｕｃｈｉら、Ｊ．Ｂｉｏｃｈｅｍ．（１９９７）１２２：７４９－７５５；Ｓａｌｉら、Ｂｉｏｃｈｅｍｉｓｔｒｙ（１９９８）３７：３３９２－３４０１；Ｃｈｏら、Ｊ．Ｖｉｒｏｌ．Ｍｅｔｈ．（１９９８）７２：１０９－１１５；Ｃｅｒｒｅｔａｎｉら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．（１９９９）２６６：１９２－１９７；Ｚｈａｎｇら、Ａｎａｌ．Ｂｉｏｃｈｅｍ（１９９９）２７０：２６８－２７５；Ｋａｋｉｕｃｈｉら、Ｊ．Ｖｉｏｌ．Ｍｅｔｈ．（１９９９）８０：７７－８４；Ｆｏｗｌｅｒら、Ｊ．Ｂｉｏｍｏｌ．Ｓｃｒｅｅｎ．（２０００）５：１５２－１５８；およびＫｉｍら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．（２０００）２８４：４２－４８を参照のこと。プロテアーゼ活性を試験するための特に便利なアッセイは、以下の実施例において記載される。

【００７０】

同様に、ヘリカーゼ活性アッセイは、当該分野で周知であり、そしてＮＳ３／４ａエピトープのヘリカーゼ活性は、例えば、以下を使用して決定され得る：例えば、Ｈｓｕら、Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍｕｎ．（１９９８）２５３：５９４－５９９に記載されるようなＥＬＩＳＡアッセイ；Ｋｙｏｎｏら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．（１９９８）２５７：１２０－１２６に記載されるようなシンチレーション近接アッセイ系（ｐｒｏｘｉｍｉｔｙ ａｓｓａｙ ｓｙｓｔｅｍ）；例えば、Ｈｉｃｈａｍら、Ａｎｔｉｖｉｒａｌ Ｒｅｓ．（２０００）４６：１８１－１９３およびＫｗｏｎｇら、Ｍｅｔｈｏｄｓ Ｍｏｌ．Ｍｅｄ．（２０００）２４：９７－１１６に記載されるようなハイスループットスクリーニングアッセイ；ならびに当該分野で公知の他のアッセイ方法。例えば、Ｋｈｕら、Ｊ．Ｖｉｏｌ．（２００１）７５：２０５－２１４；Ｕｔａｍａら、Ｖｉｒｏｌｏｇｙ（２０００）２７３：３１６－３２４；Ｐａｏｌｉｎｉら、Ｊ．Ｇｅｎ．Ｖｉｏｌ．（２０００）８１：１３３５－１３４５；Ｐｒｅｕｇｓｃｈａｔら、Ｂｉｏｃｈｅｍｉｓｔｒｙ（２０００）３９：５１７４－５１８３；Ｐｒｅｕｇｓｃｈａｔら、Ｍｅｔｈｏｄｓ Ｍｏｌ．Ｍｅｄ．（１９９８）１９：３５３－３６４；ならびにＨｅｓｓｏｎら、Ｂｉｏｃｈｅｍｉｓｔｒｙ（２０００）３９：２６１９－２６２５を参照のこと。

【００７１】

抗原の長さは、免疫反応性コンホメーションエピトープ（ｃｏｎｆｏｒｍａｔｉｏｎａｌ ｅｐｉｔｏｐｅ）を維持するのに十分である。しばしば、使用される抗原を含むポリペプチドは、ほとんど全長であるが、このポリペプチドはまた、例えば、可溶性を増加させるためか、または分泌を改善するために切断され得る。一般に、ＮＳ３／４ａ中に見出されるコンホメーションエピトープは、細胞中で組換えポリペプチドとして発現され、そしてこのポリペプチドは、以下で詳細に記載されるような所望の形態のエピトープを提供す

る。

【００７２】

ＮＳ３／４ａポリペプチドに関する代表的なアミノ酸配列は、図３および図４Ａ～４Ｄに示される。図３の１８２位に存在する太字のアラニンは、別に存在し得る分子の自己触媒を防止するために、この位置において見出されるネイティブなセリンと置換される。図４Ａ～４Ｄの２位～６８６位に示されるアミノ酸配列は、ＨＣＶ－１のアミノ酸の１０２７位～１７１１位に対応する。Ｍｅｔをコードする開始コドン（ＡＴＧ）は、１位として示される。さらに、ＨＣＶ－１の１４２８位（図４のアミノ酸の４０３位）に通常存在するＴｈｒは、Ｐｒｏに変異され、ＨＣＶ－１の１４２９位（図４のアミノ酸の４０４位）に通常存在するＳｅｒは、Ｉｌｅに変異される。しかし、このエピトープが、プロテアーゼ活性および必要に応じてヘリカーゼ活性が保持されるように、そのネイティブコンホメーションを保持または回復する方法を使用して産生される限り、Ｎ末端にＭｅｔを有するか、もしくは有さないネイティブ配列、Ｎ末端にＭｅｔを有するか、もしくは有さない描写アナログ（ｄｉｐｉｃｔｅｄ　ａｎａｌｏｇ）、または他のアナログおよびフラグメントのいずれかは、本発明のアッセイにおいて使用され得る。Ｄａｓｍａｈａｐａｔｒａら、米国特許第５，８４３，７５２号およびＺｈａｎｇら、米国特許第５，９９０，２７６号（これらの両方は、ＮＳ３／４ａのアナログを記載する）。

【００７３】

ＮＳ３／４ａのＮＳ３プロテアーゼは、ＨＣＶ－１に対して番号付けされた約１０２７位～約１２０７位（図４の２位～１８２位）において見出される。ＮＳ３プロテアーゼの構造および活性部位は、公知である。例えば、Ｄｅ　Ｆｒａｎｃｅｓｃｏら、Ａｎｔｉｖｉｒ．Ｔｈｅｒ．（１９９８）３：９９－１０９；Ｋｏｃｈら、Ｂｉｏｃｈｅｍｉｓｔｒｙ（２００１）４０：６３１－６４０を参照のこと。通常、耐性であるネイティブ配列に対する変化は、分子の活性部位の以外の変化である。特に、図４のアミノ酸１位～１５５位または２位～１５５位を保持し、ほとんど置換されないか、または保存的置換のみされることが所望される。１５５を超えて存在するアミノ酸は、より大きな変化に耐性である。さらに、図４に見出されるＮＳ３／４ａ配列のフラグメントが使用される場合、これらのフラグメントは、一般に、Ｎ末端にＭｅｔを有するか、または有さない、少なくともアミノ酸１位～１５５位または２位～１５５位、好ましくは、アミノ酸１位～１７５位または２位～１７５位、そして最も好ましくは、アミノ酸１位～１８２位または２位～１８２位を含む。このヘリカーゼドメインは、ＨＣＶ－１の約１１９３位～約１６５７位（図４の２０７位～６３２位）に見出される。従って、ヘリカーゼ活性が所望される場合、この分子の位置は、ほとんど変化しないか、または保存的変化のみされた状態で維持される。当業者は、ＮＳ３／４ａの既知の構造に基づく変化に耐える他の領域を容易に決定し得る。

【００７４】

固体支持体はまた、他の抗原を含み得る。例えば、国際公開番号ＷＯ９７／４４６９に記載されるような、複数のエピトープ融合抗体（「ＭＥＦＡ」と呼ばれる）を、本発明のアッセイでの使用のために固体支持体に結合し得る。このようなＭＥＦＡは、図１および表１に示される種々のウイルス領域のうち２種以上から誘導される複数のエピトープを含む。特に、図１および表１に示されるように、ＨＣＶポリタンパク質の切断により、NH_2－コア－Ｅ１－Ｅ２－ｐ７－ＮＳ２－ＮＳ３－ＮＳ４ａ－ＮＳ４ｂ－ＮＳ５ａ－ＮＳ５ｂ－ＣＯＯＨの順序で、少なくとも１０個の異なる産物を産生する。このコアポリペプチドは、ＨＣＶ－１に対して番号付けされた、１位～１９１位に存在する（ＨＣＶ－１ゲノムに関して、Ｃｈｏｏら（１９９１）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ８８：２４５１－２４５５を参照のこと）。このポリペプチドは、さらに、アミノ酸約１位～約１７３位を含むＨＣＶポリペプチドを産生するように処理される。このエンベロープポリペプチド（Ｅ１およびＥ２）は、それぞれ、約１９２位～約３８３位および約３８４位～約７４６位に存在する。Ｐ７ドメインは、約７４７位～約８０９位に見出される。ＮＳ２は、タンパク質分解活性を有する完全な膜タンパク質であり、そしてこのポリタンパク質の約８１０位～約１０２６位に見出される。ＮＳ２は、単独か、またはＮＳ３（約

１０２７位～約１６５７位に見出される）と組み合わせてかのいずれかで、ＮＳ２－ＮＳ３単結合（ｓｉｓｓｌｅ　ｂｏｎｄ）を切断し、次に、ＮＳ３　Ｎ末端を生成し、そしてセリンプロテアーゼ活性およびＲＮＡヘリカーゼ活性の両方を含む大きなポリタンパク質を放出する。このＮＳ３プロテアーゼ（約１０２７位～約１２０７位に見出される）は、残りのポリタンパク質を処理するのに役立つ。ヘリカーゼ活性は、約１１９３位～約１６５７位に見出される。ポリタンパク質の成熟の完了は、ＮＳ３セリンプロテアーゼにより触媒される、ＮＳ３－ＮＳ４ａ結合の自己触媒による切断より開始される。ＨＣＶポリタンパク質の続くＮＳ－３媒介切断は、別のポリペプチドのＮＳ３分子による、ポリタンパク質の切断部の認識に関与するようである。これらの反応において、ＮＳ３は、ＮＳ３補因子（約１６５８位～約１７１１位に見出されるＮＳ４ａ）、２個のタンパク質（約１７１２位～約１９７２位に見出されるＮＳ４ｂ、および約１９７３位～約２４２０位に見出されるＮＳ５ａ）、ならびにＲＮＡ依存性ＲＮＡポリメラーゼ（約２４２１位～約３０１１位に見出されるＮＳ５ｂ）を遊離する。

【００７５】

【表１】

表1

ドメイン	概算の境界　*
C(コア)	1-191
E1	192-383
E2	384-746
P7	747-809
NS2	810-1026
NS3	1027-1657
NS4a	1658-1711
NS4b	1712-1972
NS5a	1973-2420
NS5b	2421-3011

＊ＨＣＶ－１に対して番号付けられている。Ｃｈｏｏら（１９９１）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ８８：２４５１－２４５５を参照のこと。

【００７６】

複数のＨＣＶ抗原は、鎖が天然には存在しないアミノ酸の単一の連続した鎖の一部である。従って、エピトープの線形順序は、エピトープが生じるゲノムにおけるそれらの線形順序とは異なる。本明細書中における使用のためのＭＥＦＡの配列の線形順序は、好ましくは、最適な抗原性のために配置される。好ましくは、これらのエピトープは、１つより多いＨＣＶ株由来であり、従って、１つのアッセイで複数のＨＣＶ株を検出する付加能力を提供する。従って、本明細書中における使用のためのＭＥＦＡは、上記のポリタンパク質由来の様々な免疫原性領域を含み得る。さらに、このポリタンパク質のコア領域におけるフレームシフトから生じるタンパク質（例えば、国際公開番号ＷＯ９９／６３９４１に記載されるようなタンパク質）は、ＭＥＦＡにおいて使用され得る。所望ならば、ＨＣＶポリタンパク質由来の１つ以上のエピトープの少なくとも２、３、４、５、６、７、８、９もしくは１０、またはそれ以上は、融合タンパク質中で生じ得る。

【００７７】
例えば、Ｅ２の超可変領域（例えば、アミノ酸３８４～４１０または３９０～４１０にわたる領域）由来のエピトープは、ＭＥＦＡ抗原に含まれ得る。特に有効なＥ２エピトープは、この領域由来のコンセンサス配列（例えば、コンセンサス配列Ｇｌｙ－Ｓｅｒ－Ａｌａ－Ａｌａ－Ａｒｇ－Ｔｈｒ－Ｔｈｒ－Ｓｅｒ－Ｇｌｙ－Ｐｈｅ－Ｖａｌ－Ｓｅｒ－Ｌｅｕ－Ｐｈｅ－Ａｌａ－Ｐｒｏ－Ｇｌｙ－Ａｌａ－Ｌｙｓ－Ｇｌｎ－Ａｓｎ（これは１型ＨＣＶゲノムのアミノ酸３９０～４１０のコンセンサス配列を表す））を含むエピトープである。本発明のＭＥＦＡに存在する代表的なＥ２エピトープは、アミノ酸３９０～４４４にわたるハイブリッドエピトープを含み得る。このようなハイブリッドＥ２エピトープは、ＨＣＶ　Ｅ２のアミノ酸４１１～４４４のネイティブのアミノ酸配列に融合したアミノ酸３９０～４１０を表すコンセンサス配列を含み得る。

【００７８】
さらに、抗原は、様々なＨＣＶ株由来であり得る。ＨＣＶの複数のウイルス株が公知であり、そしてこれらの株のいずれか由来のエピトープは、融合タンパク質において使用され得る。生物体の任意の所定の種は、個々の生物体ごとに異なり、さらにウイルスのような所定の生物体は多数の異なる株を有することが周知である。例えば、上で説明されたように、ＨＣＶは、少なくとも６個の遺伝型を含む。これらの遺伝型の各々は、等価な抗原決定基を含む。より詳細には、各株は、ウイルスの全ての株に存在するが、ウイルス株ごとにわずかに異なる多数の抗原決定基を含む。例えば、ＨＣＶは、５－１－１として公知の抗原決定基を含む（図１を参照のこと）。この特定の抗原決定基は、ＨＣＶの３つの異なるウイルス株上で３つの異なる形態で現れる。従って、本発明の好ましい実施形態において、５－１－１の３つ全ての形態は、目的のイムノアッセイにおいて使用される多エピトープ融合抗原上に現れる。同様に、異なるＨＣＶ株のコア領域由来の等価な抗原決定基がまた存在し得る。一般的に、等価な抗原決定基は、アミノ酸配列に関して高度な相同性を有し、この相同性の程度は、整列された場合、一般に３０％以上、好ましくは４０％以上である。本発明の多コピーエピトープはまた、同じエピトープの正確なコピーである複数のコピーを含み得る。

【００７９】
本発明のアッセイと共に使用するための代表的なＭＥＦＡは、国際公開番号ＷＯ９７／４４４６９に記載される。本明細書中で使用するためのさらなる代表的なＭＥＦＡとしては、ＭＥＦＡ１２、ＭＥＦＡ１３、およびＭＥＦＡ１３．１と呼ばれるＭＥＦＡが挙げられる。これらのＭＥＦＡは、単に例示であり、そしてＨＣＶゲノム由来の他のエピトープもまた本発明のアッセイを用いる用途を見出し、そしてこれらまたは他のＭＥＦＡに組み込まれえることが理解されるべきである。

【００８０】
ＭＥＦＡ１２のＤＮＡ配列および対応するアミノ酸配列を、図７Ａ～７Ｆに示す。ＭＥＦＡ１２の一般構造式は、図６に示され、そして以下のとおりである：ｈＳＯＤ－Ｅ１（タイプ１）－Ｅ２　ＨＶＲコンセンサス（タイプ１ａ）－Ｅ２　ＨＶＲコンセンサス（タイプ１および２）－ｃ３３ｃショート（タイプ１）－５－１－１（タイプ１）５－１－１（タイプ３）－５－１－１（タイプ２）－ｃ１００（タイプ１）－ＮＳ５（タイプ１）－ＮＳ５（タイプ１）－コア（タイプ１＋２）－コア（タイプ１＋２）。この多コピーエピトープは、ＨＣＶ－１に関して番号付けされた以下のアミノ酸配列を含む（以下に記載されるアミノ酸配列の番号付けは、Ｃｈｏｏら（１９９１）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　８８：２４５１－２４５５で提供される番号付けの指示に従い、ここでアミノ酸＃１は、コア領域のコード配列によりコードされる１番目のメチオニンである）：スーパーオキシドジスムターゼ（ＳＯＤ、タンパク質の組換え発現を促進するために使用される）のアミノ酸１～６９；Ｅ１領域由来のポリタンパク質のアミノ酸３０３～３２０；ＨＣＶ－１ａ　Ｅ２の超可変領域のコンセンサス配列を表すポリタンパク質のアミノ酸３９０～４１０；ＨＣＶ－１およびＨＣＶ－２のＥ２超可変領域のコンセンサス配列を表す、領域Ｅ２由来のポリタンパク質のアミノ酸３８４～４１４；ヘリカーゼを規定する

ＨＣＶ－１ポリタンパク質のアミノ酸１２１１～１４５７；５－１－１のアミノ酸１６８９～１７３５由来のエピトープの３つのコピー（２つはＨＣＶ－１由来であり、１つはＨＣＶ－３由来であり、そして１つはＨＣＶ－２由来であり、これらのコピーは、ＨＣＶの３つの異なるウイルス株由来の等価な抗原決定基である）；ＨＣＶ－１のＨＣＶポリペプチドＣ１００である、ポリタンパク質のアミノ酸１９０１～１９３６；ＨＣＶ－１のＮＳ５領域由来のエピトープの２つの正確なコピー（この各々はＨＣＶポリタンパク質のアミノ酸２２７８～２３１３を有する）；およびコア領域由来の３つのエピトープの２つのコピー（１つはＨＣＶ－１由来であり、１つはＨＣＶ－２由来であり、これらのコピーは、ＨＣＶ－１のアミノ酸９～５３および６４～８８、ならびにＨＣＶ－２のアミノ酸６７～８４により表される等価な抗原決定基である）。

【００８１】

表２は、本明細書中の図７Ａ～７Ｆを参照して、ＭＥＦＡ１２中の様々なエピトープのアミノ酸位置を示す。この表における番号付けは、ＨＣＶ－１に関する。Ｃｈｏｏら（１９９１）Ｐｒｏｃ．Ｎａｔｌ．Ｓｃｉ．ＵＳＡ ８８：２４５１－２４５５を参照のこと。ＭＥＦＡ１３および１３．１はまた、それぞれ、表３および４に示されるような改変を有する、ＭＥＦＡ１２について上で特定された一般式を共有する。

【００８２】

【表２】

表 2. MEFA 12

mefa aa#	5' 末端部位	エピトープ	hcv aa#	株
1-69*	*Nco*1	hSOD		
72-89	*Mlu*I	E1	303-320	1
92-112	*Hind*111	E2 HVR1a コンセンサス	390-410	1
113-143		E2 HVR1+2 コンセンサス	384-414	1, 2
146-392	*Spe*I	C33C ショート	1211-1457	1
395-441	*Sph*I	5-1-1	1689-1735	1
444-490	*Nru*I	5-1-1	1689-1735	3
493-539	*Cla*I	5-1-1	1689-1735	2
542-577	*Ava*I	C100	1901-1936	1
580-615	*Xba*I	NS5	2278-2313	1
618-653	*Bgl*II	NS5	2278-2313	1
654-741	*Nco*I	コア エピトープ	9-53, R47L 64-88 67-84	1 1 2
742-829	*Bal*I	コア エピトープ	9-53, R47L 64-88 67-84	1 1 2

＊ＳＯＤタンパク質は、検出結合体であるＨＲＰ標識抗ＳＯＤ抗体がＭＥＦＡに結合しな

いように切断される。コアエピトープは、検出に使用されるＨＣＶコアに対する抗体がＭＥＦＡに結合しないように変異される。

【００８３】

【表３】

表　3. MEFA 13

mefa aa#	5' 末端部位	エピトープ	hcv aa#	株
1-156	*Nco*1	変異した hSOD (aa 70-72, ALA)		
161-178	*Mlu*I	E1	303-320	1
181-201	*Hind*111	E2 HVR1a コンセンサス	390-410	1
202-232		E2 HVR1+2 コンセンサス	384-414	1, 2
235-451		C33C ショート	1211-1457	1
454-500	*Hind*III	5-1-1 PImut*	1689-1735	1
503-549	*Nru*I	5-1-1 PImut*	1689-1735	3
552-598	*Cla*I	5-1-1 PImut*	1689-1735	2
601-636	*Ava*I	C100	1901-1936	1
639-674	*Xba*I	NS5	2278-2313	1
677-712	*Bgl*II	NS5	2278-2313	1
713-800		コア エピトープ	9-53, R47L 64-88 67-84	1 1 2
801-888		コア エピトープ	9-53, R47L 64-88 67-84	1 1 2

＊５－１－１エピトープは、ＮＳ３／４ａ組換えタンパク質によって標的化される可能な切断部位（ＣＳまたはＣＡ）を除去することによって改変される。ＣＳまたはＣＡの代わりに、この配列はＰＩに変えられる。さらに、ＳＯＤタンパク質は、検出結合体であるＨＲＰ標識抗ＳＯＤ抗体がＭＥＦＡに結合しないように変異される。コアエピトープは、検出に使用されるＨＣＶに対する抗体がＭＥＦＡに結合しないように変異される。

【００８４】

【表４】

表 4. MEFA 13.1

mefa aa#	5'末端部位	エピトープ	hcv aa#	株
1-86	*Nco*I	変異した hSOD (aa 70-72, ALA)		
89-106	*Mlu*I	E1	303-320	1
109-129	*Hind*III	E2 HVR1a コンセンサス	390-410	1
130-160		E2 HVR1+2 コンセンサス	384-414	1, 2
163-379		C33Cショート	1211-1457	1
382-428	*Hind*III	5-1-1 PImut*	1689-1735	1
431-477	*Nru*I	5-1-1 PImut*	1689-1735	3
480-526	*Cla*I	5-1-1 PImut*	1689-1735	2
529-564	*Ava*I	C100	1901-1936	1
567-602	*Xba*I	NS5	2278-2313	1
605-640	*Bgl*II	NS5	2278-2313	1
641-728		コアエピトープ	9-53, R47L 64-88 67-84	1 1 2
729-816		コアエピトープ	9-53, R47L 64-88 67-84	1 1 2

＊５－１－１エピトープは、ＮＳ３／４ａ組換えタンパク質によって標的化される可能な切断部位（ＣＳまたはＣＡ）を除去することによって改変される。ＣＳまたはＣＡの代わりに、この配列はＰＩに変えられる。さらに、ＳＯＤタンパク質は、検出結合体であるＨＲＰ標識抗ＳＯＤ抗体がＭＥＦＡに結合しないように変異される。コアエピトープは、検出に使用されるＨＣＶコアに対する抗体がＭＥＦＡに結合しないように変異される。

【００８５】

１つのアッセイ様式において、サンプルは、以下にさらに記載されるように、固体支持体と合わされる。サンプルがＨＣＶに感染している場合、コア抗原およびこの固体支持体上に存在するこれらのエピトープに対するＨＣＶ抗体は、固体支持体成分に結合する。次いで、検出可能に標識された抗コア抗体が、加えられる。この標識された抗コア抗体は、固体支持体に結合する抗コア抗体とは異なるエピトープに対して指向される。この抗コア抗体は、固体支持体上の抗コア抗体により捕捉されるコア抗原に結合する。

【００８６】

生物学的サンプル由来の捕捉されたＨＣＶ抗体（この捕捉されたサンプルのＨＣＶ抗体はＮＳ３／４ａエピトープと反応性である）と反応する抗原もまた加えられる。この抗原は、好ましくは、ＨＣＶポリタンパク質のＮＳ３領域由来のエピトープである。この抗原は、サンプル由来の捕捉されたＨＣＶ抗体に結合する。このようなエピトープを含む多数の

抗原が公知であり、これには、ｃ３３ｃおよびｃ１００領域由来の抗原、およびＮＳ３エピトープ（例えば、ｃ２５）を含む融合タンパク質が挙げられるが、これらに限定されない。これらおよび他のＮＳ３エピトープは、本発明のアッセイにおいて有用であり、そして当該分野で公知であり、そして例えば、以下に記載されている：Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号；Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９２）８９：１００１１－１００１５；Ｃｈｉｅｎら、Ｊ．Ｇａｓｔｒｏｅｎｔ．Ｈｅｐａｔｏｌ．（１９９３）８：Ｓ３３－３９；Ｃｈｉｅｎら、国際公開番号ＷＯ９３／００３６５；Ｃｈｉｅｎ，Ｄ．Ｙ．，国際公開番号ＷＯ９４／０１７７８；ならびに共有に係る特許になった米国特許出願番号０８／４０３，５９０および同０８／４４４，８１８。

【００８７】

上記の抗原に対する第２の標識された抗体が加えられる。この抗体は、抗原に含まれる任意のエピトープに対して指向され得る。例えば、この抗体は、抗原中に存在するＮＳ３領域に対して指向され得る。あるいは、上記の抗原が融合タンパク質として発現される場合、第２の標識された抗原は、融合パートナーに対して指向され得る。特に、固体支持体がＭＥＦＡを含む場合、さらなる抗原および抗体がアッセイに加えられ得る。これらのアッセイ様式は、以下にさらに説明される。

【００８８】

本発明の代表的なアッセイを図２に示す。この図に示されるように、固体支持体は、２つの抗コアモノクローナル抗体（ｃ１１－３およびＣ１１－７と呼ばれる）を含む。これらの抗体は、アミノ酸１０～５３（ＨＣＶ１ポリタンパク質配列に関して番号付けした）のコアタンパク質のＮ末端領域に見出されるエピトープに対して指向される。この固体支持体はまた、ＮＳ３／４ａに対するエピトープを含む。生物学的サンプルが、この固体支持体に加えられる。ＨＣＶコア抗原、およびＮＳ３／４ａエピトープに対する抗体（この両方はサンプル中に存在する）は、固体支持体上の捕捉試薬に結合する。

【００８９】

西洋ワサビペルオキシダーゼ（ＨＲＰ）－標識抗コアモノクローナル抗体ｃ１１－１４（アミノ酸の１２０～１３０位に見出されるコアのＣ末端領域に対する抗体、ＨＣＶ１ポリタンパク質配列に関して番号付けされている）が、加えられる。ヒトＳＯＤ（ｈＳＯＤ）由来の配列およびｃ３３ｃ領域由来のエピトープを含む融合タンパク質が、この融合タンパク質のＳＯＤ部分に対する第２のＨＲＰ標識抗体と同様に、加えられる。ＳＯＤ－ｃ３３ｃ融合タンパク質は、抗ＮＳ３抗体に結合し、そして抗ＳＯＤ抗体は、次いで、ＳＯＤ－ｃ３３ｃ融合タンパク質に結合する。標識の検出は、ＨＣＶ感染の存在を表す。

【００９０】

本発明の別の代表的なアッセイを図８に示す。抗体アッセイの構成は、ＮＳ３／４ａおよびＭＥＦＡ１２の両方を使用する抗原－抗体－抗原サンドイッチ捕捉アッセイである。固体支持体は、上記の２つの抗コアモノクローナル抗体、ＮＳ３／４ａに対するエピトープ、および代表的なＭＥＦＡであるＭＥＦＡ１２（これは、ヒトＳＯＤの短縮型を含む）を含む。上記のアッセイのように、生物学的サンプルがこの固体支持体に加えられる。ＨＣＶコア抗原、ならびにＮＳ３／４ａエピトープおよびＭＥＦＡのエピトープに対する抗体は、サンプル中に存在し、これは固体支持体上の捕捉試薬に結合する。２つの抗原（１つは、ＮＳ３／４ａに結合するサンプル抗体（上記のような）と反応性であり、そして１つは、ＭＥＦＡ１２に結合するサンプル抗体と反応性である）が添加される。図８において、ＭＥＦＡ１２／サンプル抗体複合体と反応性の抗原は、ＳＯＤ分子とｃ２２ｋｓΔ４７－Ｌ４４Ｗとの間の融合物である。このｃ２２ｋｓ抗原はコア領域由来であり、そしてポリタンパク質のアミノ酸$Ｌｙｓ_{１０}$～$Ｓｅｒ_{９９}$、ならびに通常存在する$Ａｒｇ_{４７}$の欠失、および４４位におけるＬｅｕのＴｒｐでの置換を含む。抗原検出結合体は、上記の第２のＨＲＰ標識モノクローナル抗ＳＯＤ抗体である。

【００９１】

上記の抗原／抗体組み合わせアッセイは、ＨＣＶコア抗原ならびにＮＳ３／４ａおよび／

またはコアに対する抗体の両方が、同じアッセイにおいて同じ支持体により検出され得る場合、特に有利である。さらに、上記のように、さらなるＨＣＶエピトープ（例えば、ｃ１００、５－１－１、ＮＳ５抗原に対するＳＯＤ融合タンパク質）、およびポリタンパク質のコア領域におけるフレームシフトから生じるタンパク質（例えば、国際公開番号ＷＯ９９／６３９４１に記載されるようなタンパク質）は、ＨＣＶの他の非構造的エピトープを網羅する組み合わせカクテルにおいて使用され得る。

【００９２】

本発明のさらなる理解のために、より詳細な議論は、本発明のイムノアッセイにおける使用のための抗体の生成、このイムノアッセイにおける使用のためのポリペプチドの生成、およびこのイムノアッセイを実施する方法に関して以下に記載される。

【００９３】

（ＨＣＶイムノアッセイにおける使用のための抗体の生成）

上で説明したように、アッセイは、固体支持体（例えば、１つ以上の抗コア抗体）に結合した種々の抗体、およびＨＣＶ感染がサンプル中に存在する場合に形成される抗原／抗体複合体を検出する種々の抗体を利用する。これらの抗体は、ポリクローナル抗体調製物またはモノクローナル抗体調製物、単一特異的抗血清、ヒト抗体であってもよいし、例えば、ヒト化抗体、変性抗体、Ｆ（ａｂ’）$_2$フラグメント、Ｆ（ａｂ）フラグメント、Ｆｖフラグメント、単一ドメイン抗体、二量体または三量体の抗体フラグメント構築物、ミニ抗体（ｍｉｎｉｂｏｄｉｅｓ）、または問題の抗原に結合するそれらの機能的フラグメントのような抗体ハイブリッド抗体またはキメラ抗体であってもよい。

【００９４】

抗体は、当業者に周知の技術、ならびに例えば、米国特許第４，０１１，３０８号；同第４，７２２，８９０号；同第４，０１６，０４３号；同第３，８７６，５０４号；同第３，７７０，３８０号；および同第４，３７２，７４５号に記載される技術を使用して生成される。例えば、ポリクローナル抗体は、適切な動物（例えば、マウス、ラット、ウサギ、ヒツジまたはヤギ）を、目的の抗原で免疫することによって生成される。免疫原性を増強するために、抗原は、免疫の前にキャリアに結合され得る。このようなキャリアは、当業者に周知である。免疫は、一般に、生理食塩水中、好ましくは、フロイントの完全アジュバントのようなアジュバント中の抗原を混合または乳化することによって、そして混合物またはエマルジョンを非経口的（一般に、皮下または筋肉内）に注射することによって行なわれる。動物は、一般に、好ましくは、フロイントの不完全アジュバントを使用して、生理食塩水中の抗原の１回以上の注射で、２～６週後に追加免疫される。抗体はまた、当該分野で公知の方法を使用する、インビトロ免疫によって生成され得る。次いで、ポリクローナル抗血清は、免疫動物から得られる。例えば、抗ＨＣＶポリクローナル抗体の生成については、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号を参照のこと。

【００９５】

モノクローナル抗体は、一般に、ＫｏｈｌｅｒおよびＭｉｌｓｔｅｉｎ（１９７５）Ｎａｔｕｒｅ　２５６：４９５－４９７の方法またはその改変物を使用して調製される。代表的には、マウスまたはラットが、上記のように免疫される。しかし、血清を抽出するために動物を出血させるのではなく、脾臓（および必要に応じていくつかの大きいリンパ節）が取り出され、そして単一の細胞に分離される。所望の場合、脾臓細胞は、抗原でコーティングしたプレートまたはウェルに細胞懸濁液を適用することによって、（非特異的に接着した細胞の除去後に）スクリーニングされ得る。Ｂ細胞（抗原に特異的な膜結合免疫グロブリンを発現する）はプレートに結合し、そして懸濁液の残りと共に洗い流されない。次いで得られたＢ細胞（または全ての分離した脾臓細胞）は、黒色腫細胞と融合するように誘導されてハイブリドーマを形成し、そして選択的な培地（例えば、ヒポキサンチン、アミノプテリン、チミジン培地、「ＨＡＴ」）で培養される。得られたハイブリドーマは、限界希釈によってプレートされ、そして、免疫抗原に特異的に結合する（そして関連していない抗原に結合しない）抗体の生成についてアッセイされる。次いで、選択されたモノクローナル抗体スクリーニングハイブリドーマは、インビトロ（例えば、組織培養瓶ま

たは中空線維反応器中）またはインビボ（例えば、マウスの腹水のような）のいずれかで培養される。

【００９６】

種々の抗ＨＣＶモノクローナル抗体の生成は、例えば、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号；Ｃｈｉｅｎら、国際公開番号ＷＯ９３／００３６５；共有に係る、特許となった米国特許出願番号０８／４０３，５９０および同０８／４４４，８１８；およびＫａｓｈｉｗａｋｕｍａら、米国特許番号５，８７１，９０４号において記載されている。

【００９７】

上に説明されたように、目的の抗原を認識する能力を保持する抗体フラグメントはまた、目的のイムノアッセイにおいて用途を見出す。インタクトな抗体分子の免疫学的結合特性を示し得る抗原結合部位を含む多数の抗体フラグメントが公知である。例えば、機能的な抗体フラグメントは、例えば、ペプシンを使用して、抗体分子から抗原結合を担わない定常領域を切断することによって生成され、Ｆ（ａｂ’）$_2$フラグメントを生成する。これらのフラグメントは、２つの抗原結合部位を含むが、重鎖の各々から定常領域の一部分を欠く。同様に、所望な場合、Ｆａｂフラグメント（単一の抗原結合部位を含む）が、例えば、パパインでのポリクローナル抗体またはモノクローナル抗体の消化によって、生成され得る。機能的フラグメント（重鎖および軽鎖の可変領域のみを含む）は、免疫グロブリン分子の組換え生成または優先的タンパク質分解切断のような標準的な技術を使用して、生成され得る。これらのフラグメントは、Ｆｖとして公知である。例えば、Ｉｎｂａｒら（１９７２）Ｐｒｏｃ．Ｎａｔ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ６９：２６５９－２６６２；Ｈｏｃｈｍａｎら（１９７６）Ｂｉｏｃｈｅｍ １５：２７０６－２７１０；およびＥｈｒｌｉｃｈら（１９８０）Ｂｉｏｃｈｅｍ １９：４０９１－４０９６を参照のこと。

【００９８】

単鎖Ｆｖ（「ｓＦｖ」または「ｓｃＦｖ」）ポリペプチドは、ペプチドコードリンカーによって連結されるV_Hコード遺伝子およびV_Lコード遺伝子を含む遺伝子融合物から発現される共有結合したV_H－V_Lヘテロ二量体である。Ｈｕｓｔｏｎら（１９８８）Ｐｒｏｃ．Ｎａｔ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：５８７９－５８８３。天然に凝集しているが化学的に分離した、抗体Ｖ領域由来の軽鎖および重鎖ポリペプチドを、抗原結合部位の構造に実質的に類似した三次元構造に折り畳まれるｓＦｖ分子に変換する化学的構造（リンカー）を区別および開発するための多数の方法が記載されている。例えば、米国特許第５，０９１，５１３号、同第５，１３２，４０５号、および同第４，９４６，７７８号を参照のこと。ｓＦｖ分子は、当該分野で記載される方法を使用して生成され得る。例えば、Ｈｕｓｔｏｎら（１９８８）Ｐｒｏｃ．Ｎａｔ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：５８７９－５８８３；米国特許第５，０９１，５１３号、同第５，１３２，４０５号、および同第４，９４６，７７８号を参照のこと。設計基準は、一方の鎖のＣ末端と、他方の鎖のＮ末端との間の距離にわたる適切な長さを決定することを包含し、ここで、リンカーは、一般に、コイル化する傾向がないか、または二次構造を形成しない親水性低分子アミノ酸残基から形成される。このような方法は、当該分野で公知である。例えば、米国特許第５，０９１，５１３号、同第５，１３２，４０５号、および同第４，９４６，７７８号を参照のこと。適切なリンカーは、一般に、代替のセットのグリシンおよびセリン残基のポリペプチド鎖を含み、そして可溶性を増強するために、挿入されたグルタミン酸およびリジン残基を含み得る。

【００９９】

「ミニ抗体（ｍｉｎｉ－ａｎｔｉｂｏｄｉｅｓ）」または「ミニ抗体（ｍｉｎｉｂｏｄｉｅｓ）」もまた、本発明を用いる用途が見出される。ミニ抗体は、ヒンジ領域によってｓＦｖから分離された、そのＣ末端にオリゴマー形成ドメインを含むｓＦｖポリペプチド鎖である。Ｐａｃｋら（１９９２）Ｂｉｏｃｈｅｍ ３１：１５７９－１５８４。オリゴマー形成ドメインは、さらなるジスルフィド結合によってさらに安定化され得る自己会合αヘリックス（例えば、ロイシンジッパー）を含む。このオリゴマー形成ドメインは、膜を

横切る特定の方向への折り畳みと適合性であるように設計され、この折り畳みは、ポリペプチドの機能的な結合タンパク質へのインビボの折り畳みを容易にすると考えられているプロセスである。一般に、ミニ抗体は、当該分野で周知の方法を使用して生成される。例えば、Packら（１９９２）Biochem ３１：１５７９－１５８４；Cumberら（１９９２）J Immunology １４９B：１２０－１２６を参照のこと。

【０１００】

（ＨＣＶイムノアッセイにおける使用のための抗原の生成）

上で説明したように、本発明の分子は、一般に、組換え的に生成される。従って、本発明と共に使用するための、ＨＣＶ抗原をコードするポリヌクレオチドは、分子生物学の標準的な技術を使用して作製され得る。例えば、上記の分子をコードするポリヌクレオチド配列は、組換え方法を使用して（例えば、この遺伝子を発現する細胞由来のｃＤＮＡおよびゲノムライブラリをスクリーニングすることによって、またはこの遺伝子を含むことが知られているベクターから遺伝子を誘導することによって）得られ得る。さらに、所望の遺伝子は、当該分野（例えば、Houghtonら、米国特許第５，３５０，６７１号）に記載される技術を使用して、ウイルス核酸分子から直接単離され得る。目的の遺伝子はまた、クローン化よりむしろ合成的に生成され得る。これらの分子は、特定の配列について適切なコドンを用いて設計され得る。次いで、完全配列は、標準的な方法により調製される重複オリゴヌクレオチドから構築され、そして完全コード配列に構築される。例えば、Edge（１９８１）Nature ２９２：７５６；Nambairら（１９８４）Science ２２３：１２９９；およびJayら（１９８４）J. Biol. Chem. ２５９：６３１１を参照のこと。

【０１０１】

従って、特定のヌクレオチド配列は、所望の配列を保有するベクターから得られ得るか、または当該分野で公知の様々なオリゴヌクレオチド合成技術（例えば、適切な場合、部位特異的変異誘発およびポリメラーゼ連鎖反応（ＰＣＲ））を使用して、完全にもしくは部分的に合成され得る。例えば、Sambrook、（前出）を参照のこと。特に、所望の配列をコードするヌクレオチド配列を得る１つの方法は、従来の自動化ポリヌクレオチド合成機で生成される重複合成オリゴヌクレオチドの相補的なセットをアニーリングし、続いて適切なＤＮＡリガーゼで連結し、そしてＰＶＲによりこの連結したヌクレオチド配列を増幅することによるものである。例えば、Jayaramanら、（１９９１）Proc. Natl. Acad. Sci. USA ８８：４０８１～４０８８を参照のこと。さらに、オリゴヌクレオチド指向型合成（Jonesら（１９８６）Nature ５４：７５～８２）、既存のヌクレオチド領域のオリゴヌクレオチド指向型変異誘発（Reichmannら（１９８８）Nature ３３２：３２３～３２７、およびVerhoeyenら（１９８８）Science ２３９：１５３４～１５３６）、およびT_4 ＤＮＡポリメラーゼを使用するギャップオリゴヌクレオチド（gapped oligonucleotide）の酵素充填（enzymatic filling－in）（Queenら（１９８９）Proc. Natl. Acad. Sci. USA ８６：１００２９～１００３３）を本発明で使用して、改変されたかもしくは増大された抗原結合能力、および／または減少した免疫原性を有する分子を提供し得る。

【０１０２】

一旦、コード配列が調製または単離されると、このような配列は、任意の適切なベクターまたはレプリコンにクローン化され得る。多数のクローニングベクターが当業者に公知であり、そして適切なクローニングベクターの選択が可能である。適切なベクターとしては、プラスミド、ファージ、トランスポゾン、コスミド、染色体または適切なコントロールエレメントと会合した場合に複製し得るウイルスが挙げられるが、これらに限定されない。

【０１０３】

次いで、コード配列は、発現のために使用される系に依存して、適切なコントロールエレメントの制御下に置かれる。従って、このコード配列は、プロモータ、リボソーム結合部

位（細菌の発現のため）、および必要に応じて、オペレーターの制御下に置かれ、その結果、目的のＤＮＡ配列は、適切な形質転換体によってＲＮＡに転写される。このコード配列は、シグナルペプチドまたはリーダー配列（これは後に、翻訳後プロセシングにおいて、宿主によって除去され得る）を含んでも含まなくても良い。例えば、米国特許第４，４３１，７３９号；同第４，４２５，４３７号；同第４，３３８，３９７号を参照のこと。

【０１０４】

制御配列に加えて、宿主細胞の増殖に関連した配列の発現の調節を可能にする調節配列を加えることが所望であり得る。調節配列は、当業者に公知であり、そして例としては、調節化合物を含む、化学的刺激または物理的刺激に対してオンまたはオフにされる遺伝子の発現を引き起こすものが挙げられる。他の型の調節エレメントはまた、ベクター中に存在し得る。例えば、エンハンサーエレメントは、構築物の発現レベルを増加するために本明細書中で使用され得る。例としては、ＳＶ４０初期遺伝子エンハンサー（Ｄｉｊｋｅｍａら（１９８５）ＥＭＢＯ Ｊ．４：７６１）、ラウス肉腫ウイルスの長末端反復（ＬＴＲ）に由来するエンハンサー／プロモーター（Ｇｏｒｍａｎら（１９８２）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ７９：６７７７）およびヒトＣＭＶに由来するエレメント（Ｂｏｓｈａｒｔら（１９８５）Ｃｅｌｌ ４１：５２１）（例えば、ＣＭＶイントロンＡ配列に含まれるエレメント（米国特許第５，６８８，６８８））が挙げられる。発現カセットはさらに、適切な宿主細胞において自律的に複製するための複製起点、１つ以上の選択マーカー、１つ以上の制限部位、高いコピー数についての可能性および強力なプロモーターを含む。

【０１０５】

発現ベクターは、特定のコード配列が適切な制御配列と共にベクター中に位置するように構築され、制御配列に対するコード配列の位置および配向は、コード配列が制御配列の「制御」下で転写されるように存在する（すなわち、制御配列のＤＮＡ分子に結合するＲＮＡポリメラーゼは、コード配列を転写する）。目的の分子をコードする配列の改変は、この目的を達成するために所望である。例えば、いくつかの場合において、適切な配向で配列が制御配列に付着するように（すなわち、リーディングフレームを維持するように）配列を改変することが必要であり得る。制御配列および他の調節配列は、ベクターへの挿入の前にコード配列に連結され得る。あるいは、コード配列は、制御配列および適切な制限部位をすでに含む発現ベクターに直接クローニングされ得る。

【０１０６】

上に説明されるように、目的の抗原の変異体またはアナログを生成することが所望であり得る。これは、特にＮＳ３／４ａに当てはまる。そうするための方法は、例えば、Ｄａｓｍａｈａｐａｔｒａら、米国特許第５，８４３，７５２号およびＺｈａｎｇら、米国特許５，９９０，２７６号において記載されている。目的のアッセイにおける使用のためのこのＨＣＶタンパク質および他のＨＣＶタンパク質の変異体またはアナログは、配列の挿入によって、そして／または配列内での１つ以上のヌクレオチドの置換によって、目的のポリペプチドをコードする配列の一部を欠失することによって調製され得る。ヌクレオチド配列を改変するための方法（例えば、部位特異的変異誘発など）は、当該分野で公知である。例えば、Ｓａｍｂｒｏｏｋら、前出；Ｋｕｎｋｅｌ，Ｔ．Ａ．（１９８５）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９８５）８２：４４８；Ｇｅｉｓｓｅｌｓｏｄｅｒら（１９８７）ＢｉｏＴｅｃｈｎｉｑｕｅｓ ５：７８６；ＺｏｌｌｅｒおよびＳｍｉｔｈ（１９８３）Ｍｅｔｈｏｄｓ Ｅｎｚｙｍｏｌ．１００：４６８；Ｄａｌｂｉｅ－ＭｃＦａｒｌａｎｄら（１９８２）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ ＵＳＡ ７９：６４０９を参照のこと。

【０１０７】

分子は、昆虫系、哺乳動物系、細菌系、ウイルス系および酵母系を含む広範な種々の系において発現され得、これらは全て当該分野で公知である。

【０１０８】

例えば、昆虫細胞発現系（例えば、バキュロウイルス系）は、当業者に公知であり、そし

て例えば、ＳｕｍｍｅｒｓおよびＳｍｉｔｈ，Ｔｅｘａｓ Ａｇｒｉｃｕｌｔｕｒａｌ Ｅｘｐｅｒｉｍｅｎｔ Ｓｔａｔｉｏｎ Ｂｕｌｌｅｔｉｎ Ｎｏ．１５５５（１９８７）において記載されている。バキュロウイルス／昆虫細胞発現系のための材料および方法は、特に、Ｉｎｖｉｔｒｏｇｅｎ，Ｓａｎ Ｄｉｅｇｏ ＣＡ（「ＭａｘＢａｃ」キット）から市販されている。同様に、細菌および哺乳動物細胞発現系は、当該分野で周知であり、そして例えば、Ｓａｍｂｒｏｏｋら、前出、において記載されている。酵母発現系はまた、当該分野で公知であり、そして例えば、Ｙｅａｓｔ Ｇｅｎｅｔｉｃ Ｅｎｇｉｎｅｅｒｉｎｇ（Ｂａｒｒら編、１９８９）Ｂｕｔｔｅｒｗｏｒｔｈｓ，Ｌｏｎｄｏｎにおいて記載されている。

【０１０９】

上の系を用いる使用のための多数の適切な宿主細胞がまた公知である。例えば、哺乳動物細胞株は、当該分野で公知であり、そして例えば、チャイニーズハムスター卵巣（ＣＨＯ）細胞、ＨｅＬａ細胞、乳児ハムスター腎（ＢＨＫ）細胞、サル腎細胞（ＣＯＳ）、ヒト胚腎細胞、ヒト肝細胞癌細胞（例えば、Ｈｅｐ Ｇ２）、Ｍａｄｉｎ－Ｄａｒｂｙウシ腎（「ＭＤＢＫ」）細胞、およびその他を含むがこれらに限定されない、Ａｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎ（ＡＴＣＣ）から入手可能な不死化細胞株を含む。同様に、Ｅ．ｃｏｌｉ、Ｂａｃｉｌｌｕｓ ｓｕｂｔｉｌｉｓ、およびＳｔｒｅｐｔｏｃｏｃｃｕｓ ｓｐｐ．のような細菌宿主において、本発明の構築物を用いる用途を見出す。本発明において有用な酵母宿主としては特に、Ｓａｃｃｈａｒｏｍｙｃｅｓ ｃｅｒｅｖｉｓｉａｅ、Ｃａｎｄｉｄａ ａｌｂｉｃａｎｓ、Ｃａｎｄｉｄａ ｍａｌｔｏｓａ、Ｈａｎｓｅｎｕｌａ ｐｏｌｙｍｏｒｐｈａ、Ｋｌｕｙｖｅｒｏｍｙｃｅｓ ｆｒａｇｉｌｉｓ、Ｋｌｕｙｖｅｒｏｍｙｃｅｓ ｌａｃｔｉｓ、Ｐｉｃｈｉａ ｇｕｉｌｌｅｒｉｍｏｎｄｉｉ、Ｐｉｃｈｉａ ｐａｓｔｏｒｉｓ、Ｓｃｈｉｚｏｓａｃｃｈａｒｏｍｙｃｅｓ ｐｏｍｂｅおよびＹａｒｒｏｗｉａ ｌｉｐｏｌｙｔｉｃａが挙げられる。バキュロウイルス発現ベクターを用いる使用のための昆虫細胞としては特に、Ａｅｄｅｓ ａｅｇｙｐｔｉ、Ａｕｔｏｇｒａｐｈａ ｃａｌｉｆｏｒｎｉｃａ、Ｂｏｍｂｙｘ ｍｏｒｉ、Ｄｒｏｓｏｐｈｉｌａ ｍｅｌａｎｏｇａｓｔｅｒ、Ｓｐｏｄｏｐｔｅｒａ ｆｒｕｇｉｐｅｒｄａおよびＴｒｉｃｈｏｐｌｕｓｉａ ｎｉが挙げられる。

【０１１０】

目的のヌクレオチド配列を含む核酸分子は、当該分野で周知の遺伝子送達技術を使用して、宿主細胞ゲノムに安定に組み込まれ得るか、または適切な宿主細胞中の安定なエピソームエレメント上に維持され得る。例えば、米国特許第５，３９９，３４６号を参照のこと。

【０１１１】

選択される発現系および宿主に依存して、分子は、タンパク質が発現される条件下で、上記の発現ベクターによって形質転換された宿主細胞を増殖することによって生成される。次いで、発現されたタンパク質は、宿主細胞から単離され、そして精製される。発現系が、タンパク質を増殖培地中に分泌する場合、生成物は、培地から直接精製され得る。生成物が分泌されない場合、生成物は細胞溶解物から単離され得る。適切な増殖条件および回収方法の選択は、当該分野の技量内である。

【０１１２】

種々のＨＣＶ抗原の組換え生成が記載されている。例えば、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号；Ｃｈｉｅｎら、Ｊ．Ｇａｓｔｒｏｅｎｔ．Ｈｅｐａｔｏｌ．（１９９３）８：Ｓ３３－３９；Ｃｈｉｅｎら、国際公開番号ＷＯ９３／００３６５；Ｃｈｉｅｎ，Ｄ．Ｙ．，国際公開番号ＷＯ９４／０１７７８を参照のこと。

【０１１３】

（免疫診断アッセイ）

一旦生成されると、上記の抗コア抗体およびＮＳ３／４ａ抗原は、本イムノアッセイにおける使用のために適切な固体支持体上に配置される。本発明の目的のための固体支持体は、不溶性マトリクスである任意の物質であり得、そして剛性または半剛性の表面を有し得

る。例示的な固体支持体としては、ニトロセルロースのような基材（例えば、膜またはマイクロタイターウェル形態）；ポリ塩化ビニル（例えば、シートまたはマイクロタイターウェル）；ポリスチレンラテックス（例えば、ビーズまたはマイクロタイタープレート）；ポリフッ化ビニリデン；ジアゾ化紙；ナイロン膜；活性化ビーズ、磁気応答性樹脂などが挙げられるがこれらに限定されない。特定の支持体としては、プレート、ペレット、ディスク、キャピラリー、中空繊維、針、ピン、固体繊維、セルロースビーズ、孔－ガラスビーズ、シリカゲル、ジビニルベンゼンと必要に応じて架橋したポリスチレンビーズ、グラフトされたコポリ（ｃｏ－ｐｏｌｙ）ビーズ、ポリアクリルアミドビーズ、ラテックスビーズ、Ｎ－Ｎ’－ビス－アクリロイルエチレンジアミンと必要に応じて架橋したジメチルアクリルアミドビーズ、および疎水性ポリマーでコーティングしたガラス粒子が挙げられる。

【０１１４】

所望される場合、固体支持体に付加される分子は、スチレン部分またはアクリレート部分を作製するために容易に官能基化され得、従って、ポリスチレン、ポリアクリレート、またはポリイミド、ポリアクリルアミド、ポリエチレン、ポリビニル、ポリジアセチレン、ポリフェニレン－ビニレン、ポリペプチド、多糖類、ポリスルホン、ポリピロール、ポリイミダゾール、ポリチオフェン、ポリエステル、エポキシ、シリカガラス、シリカゲル、シロキサン、ポリホスフェート、ヒドロゲル、アガロース、セルロースなどの他のポリマーへの分子の取り込みを可能にする。

【０１１５】

１つの状況において、固体支持体を、分子が十分に支持体に固定されるような適切な結合条件下で、まずＨＣＶ抗コア抗体およびＮＳ３／４ａエピトープ（本明細書中で集合的に「固相成分」と呼ばれる）、および必要に応じて１つ以上のＭＥＦＡと反応させる。時々、支持体への固定は、より優れた固相結合特性を有するタンパク質への抗原および／または抗体を最初に結合することによって強化され得る。適切なカップリングタンパク質としては、ウシ血清アルブミン（ＢＳＡ）を含む血清アルブミン、キーホールリンペットヘモシニアン、免疫グロブリン分子、チログロブリン、オボアルブミンのような高分子、および当業者に周知の他のタンパク質が挙げられるがこれらに限定されない。支持体に分子を結合させるために使用され得る他の試薬としては、多糖類、ポリ乳酸、ポリグリコール酸、ポリマー性アミノ酸、アミノ酸コポリマーなどが挙げられる。このような分子および抗原にこれらの分子を結合させる方法は、当業者に周知である。例えば、Ｂｒｉｎｋｌｅｙ，Ｍ．Ａ．（１９９２）Ｂｉｏｃｏｎｊｕｇａｔｅ Ｃｈｅｍ．３：２－１３；Ｈａｓｈｉｄａら（１９８４）Ｊ．Ａｐｐｌ．Ｂｉｏｃｈｅｍ．６：５６－６３；ならびにＡｎｊａｎｅｙｕｌｕおよびＳｔａｒｏｓ（１９８７）Ｉｎｔｅｒｎａｔｉｏｎａｌ Ｊ．ｏｆ Ｐｅｐｔｉｄｅ ａｎｄ Ｐｒｏｔｅｉｎ Ｒｅｓ．３０：１１７－１２４を参照のこと。

【０１１６】

固体支持体を固相成分と反応させた後、任意の非固定固相成分を、洗浄することによって支持体から取り除き、次いで支持体結合成分を、適切な結合条件下で、ＨＣＶ抗体および抗原（本明細書中で集合的に「リガンド分子」と呼ばれる）を含むことが疑われている生物学的サンプルと接触させる。任意の結合していないリガンド分子を除去するために洗浄した後、支持体に結合した抗コア抗体と異なるエピトープに対して指向する第２の抗コア抗体を、適切な結合条件下で添加する。添加される抗コア抗体は、上記のように、検出可能な標識を含み、そして支持体に結合した抗コア抗体と反応したサンプル中に存在し得る任意のコア抗原を結合するように作用する。ＮＳ３／４Ａエピトープと順番に反応したサンプル中に存在する抗体と反応し得る１つ以上の抗原もまた、添加される。上記で説明したように、抗原は、代表的に、ＨＣＶポリタンパク質のＮＳ３領域から誘導され、特に、ＨＣＶのｃ３３ｃ領域から誘導される。これらから誘導されるエピトープの領域の説明に関しては、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号；Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ（１９８９）８９：１００１１－１００１５；国際公

開番号ＷＯ９３／００３６５；ならびに、一般に所有された、許可された米国特許出願第０８／４０３，５９０および０８／４４４，８１８を参照のこと。この抗原に対する標識抗体もまた、添加される。従って、抗体は、サンプル中に存在する抗ＮＳ３抗体と反応した抗原に結合する。この目的のために、ｃ３３ｃエピトープは、ｃ３３ｃとヒトスーパーオキシドジスムターゼ（ｈＳＯＤ）との間の融合として好都合に提供され、例えば、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号に記載される方法によって組換え産生され得る。ヒトＳＯＤのヌクレオチド配列およびアミノ酸配列は、公知であり、Ｈａｌｌｅｗｅｌｌら、米国特許第５，７１０，０３３号に報告される。従って、ヒトＳＯＤに対する標識抗体は、ＮＳ３／４ａエピトープ、このエピトープと反応するサンプル中の任意の抗体、およびサンプル中のその抗体と順に結合するポリペプチドの間で形成される複合体の存在を検出するために使用され得る。

【０１１７】

ＭＥＦＡが固体支持体上に存在する場合、ＭＥＦＡ上に存在する抗原に結合する生物学的サンプル由来の抗体と反応性である１つ以上のさらなる抗原もまた、アッセイに添加され得る。この状況において特に有用なことは、ＨＣＶのコア領域由来の抗原であり、より詳細には、ＨＣＶポリタンパク質の１１９Ｎ末端コアアミノ酸を含むｃ２２抗原由来であることである。ｃ２２由来の１つの特定の抗原は、ポリタンパク質のアミノ酸Lys_{10}～Ser_{99}ならびに通常存在するＡｒｇ４７の欠失および４４位にＴｒｐの代わりのＬｅｕを含む、ｃ２２ｋｓ　Δ４７－Ｌ４４Ｗである。上記のｃ３３ｃエピトープに関して、この抗原は、ｈＳＯＤおよびヒトＳＯＤに対するその標識抗体との融合として提供され、サンプル中に存在する抗体と、ＮＳ３／４ａエピトープおよび／またはＭＥＦＡとの間に形成される複合体（これらの複合体はまた、ＨＣＶ抗原（例えば、ｃ３３ｃおよびｃ２２）と結合する）の存在を検出するために使用され得る。

【０１１８】

より詳細には、ＥＬＩＳＡ方法を、使用し得、ここで、マイクロタイタープレートのウェルを、固相成分と接触させる。次いで、リガンド分子を含むかまたは含むことが疑われる生物学的サンプルを、コーティングされたウェルに添加する。リガンド－分子の固定化された固相成分への結合を可能にするのに十分なインキュベーション期間の後、プレートを洗浄して、未結合の部分を除去し得、そして、検出可能に標識された２次結合分子（標識抗コア抗体）、ＮＳ３エピトープ含有分子、およびＮＳ３エピトープ含有部分に対する抗体を、添加される。これらの分子を、任意の捕獲サンプル抗原および抗体と反応させ、プレートを洗浄し、そして標識抗体の存在が、当該分野で周知の方法を用いて検出される。

【０１１９】

上記のアッセイ試薬（抗体および抗原を有するイムノアッセイ固体支持体、ならびに捕獲サンプルと反応する抗体および抗原を含む）をまた、適切な指示書および他の必要な試薬と共にキット中に提供して、上記のようにイムノアッセイを実行し得る。このキットはまた、使用する特定のイムノアッセイに依存して、適切な標識ならびに他のパッケージングされた試薬および材料（すなわち、洗浄緩衝液など）を含み得る。標準的なイムノアッセイ（例えば、上記に記載されるもの）を、これらのキットを使用して実行し得る。

【０１２０】

（ＩＩＩ．実験）

以下は、本発明を実行するための特定の実施形態の例である。本実施例は、例示目的のみのために提供され、そして本発明の範囲をいずれにも限定することは意図されない。

【０１２１】

使用した数値（例えば、量、温度など）に関して精度を確実にするための努力がなされたが、いくらかの実験誤差および偏差は、当然許容されるべきである。

【０１２２】

（実施例１）

（ＨＣＶ抗原／抗体の組み合わせイムノアッセイ）

本発明のＨＣＶ抗原／抗体の組み合わせイムノアッセイを、セロコンバージョン検出限界

を試験し、そしてこれらの限界を以下のような他の市販で入手されるアッセイに対する限界と比較するために、他のＨＣＶアッセイと比較した。

【０１２３】

（Ａ．材料および方法）

（血液サンプル）市販のヒト血液サンプルのパネルを使用した。このようなパネルは、例えば、Ｂｏｓｔｏｎ Ｂｉｏｍｅｄｉｃａ，Ｉｎｃ．，Ｗｅｓｔ Ｂｒｉｄｇｅｗａｔｅｒ，ＭＡ（ＢＢＩ）；Ｂｉｏｃｌｉｎｉｃａｌ Ｐａｒｔｎｅｒｓ，Ｆｒａｎｋｌｉｎ，ＭＡ（ＢＣＰ）；およびＮｏｒｔｈ Ａｍｅｒｉｃａｎ Ｂｉｏｌｏｇｉｃｓ，Ｉｎｃ．，ＢｏｃｏＲａｔａｎ，ＦＬ（ＮＡＢＩ）から入手可能である。表５および６に示した日は、血液を被験体から収集した日である。

【０１２４】

（モノクローナル抗体）モノクローナル抗体ｃ１１－３、ｃ１１－７およびｃ１１－１４を、Ｏｒｔｈｏ Ｃｌｉｎｉｃａｌ Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ Ｊｅｒｓｅｙから入手した。ｃ１１－３およびｃ１１－７抗体は、コアのＮ末端部分（ＨＣＶ１ポリペプチドに対する番号付けでアミノ酸１０～５３）に対して指向する。ｃ１１－１４モノクローナル抗体は、コアのＣ末端部分（ＨＣＶ１ポリペプチドに対する番号付けでアミノ酸１２０～１３０）に対して指向する。ｃ１１－１４抗体は、標準的な手順を用いて西洋ワサビペルオキシダーゼ（ＨＲＰ）に結合体化されていた。

【０１２５】

モノクローナル抗体５Ａ－３は、ＳＯＤのアミノ酸１～６５に対して指向された抗ＳＯＤ抗体であり、標準的な技術を使用して作製された。この抗体は、上記のようにＨＲＰに結合体化されていた。

【０１２６】

（Ｂ．抗原）

ｃ３３ｃ抗原（２６６アミノ酸、ＨＣＶ１ポリタンパク質のアミノ酸１１９２～１４５７）を、５－１－１抗原の合成について記載された方法（Ｃｈｏｏら、Ｓｃｉｅｎｃｅ（１９８９）２４４：３５９－３６２）によって、Ｅ．ｃｏｌｉ中で内部ＳＯＤ融合ポリペプチドとして発現させた。組換え抗原を、Ｃｈｉｅｎら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．（１９８９）８９：１００１１－１００１５に記載されるように精製した。ＳＯＤ－ｃ３３ｃの生産手順については、Ｈｏｕｇｈｔｏｎら、米国特許第５，３５０，６７１号もまた、参照のこと。

【０１２７】

本アッセイに使用されるＮＳ３／４ａエピトープは、図３に特定される配列を有するコンホメーションエピトープである。

【０１２８】

（Ｃ．イムノアッセイ形式）

Ａｂｂｏｔｔ ＰＲＩＳＭアッセイ（Ａｂｂｏｔｔ Ｌａｂｏｒａｔｏｒｉｅｓ，Ａｂｂｏｔｔ Ｐａｒｋ，ＩＬ）は、市販され、そして抗体に基づく検出アッセイである。このアッセイを、製造者らの指示書を用いて実行した。

【０１２９】

ＯＲＴＨＯ ＨＣＶ バージョン ３．０ ＥＬＩＳＡ試験システム（本明細書中、Ｏｒｔｈｏ ３．０アッセイと称する、Ｏｒｔｈｏ Ｃｌｉｎｉｃａｌ Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ Ｊｅｒｓｅｙ）は、抗体に基づく検出アッセイである。このアッセイを、製造者らの指示書を用いて実行した。

【０１３０】

Ｒｏｃｈｅ Ａｍｐｌｉｃｏｒアッセイ（Ｒｏｃｈｅ，Ｐｌｅａｓａｎｔ，ＣＡ）は、市販のＰＣＲに基づくアッセイである。このアッセイを、製造者らの指示書を用いて実行した。

【０１３１】

Ｇｅｎ－Ｐｒｏｂｅ ＴＭＡアッセイ（Ｓａｎ Ｄｉｅｇｏ，ＣＡ）は、市販の転写媒介

増幅アッセイである。このアッセイを、製造者らの指示書を用いて実行した。

【０１３２】

Ｏｒｔｈｏ抗原アッセイ（Ｏｒｔｈｏ　Ｃｌｉｎｉｃａｌ　Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ　Ｊｅｒｓｅｙ）は、抗原に基づく検出アッセイである。このアッセイを、製造者らの指示書を用いて実行した。

【０１３３】

本ＨＣＶ抗原／抗体の組み合わせイムノアッセイを、以下のように実行した。１×リン酸緩衝化生理食塩水（ＰＢＳ）（ｐＨ７．４）中、各々４ｍｇ／ｍＬの精製モノクローナル抗体Ｃ１１－７とＣ１１－３とを合わせ、そしてウェルを混合した。９０ｎｇのＮＳ３／４ａ組換え抗原を、同じコーティング緩衝液に添加した。この溶液は、コーティング前に３０分間混合した。２００ｍＬの上記の溶液を、９６ウェル　Ｃｏｓｔａｒ培地結合マイクロタイタープレート（Ｃｏｒｉｎｇ，Ｉｎｃ．）へ各ウェルにつき添加した。プレートを、１５～３０℃で１６～２４時間インキュベートした。プレートを、dH_2Oを用いて２回洗浄し、続いて、３００μＬ／ウェル　後コーティング緩衝液（１％　ウシ血清アルブミン（ＢＳＡ），１×ＰＢＳ）を用いて１時間洗浄し、そして３００μＬ／ウェルの安定緩衝液（１×ＰＢＳ，１％　ＢＳＡ，マンニトール、ポリエチレングリコール（ＰＥＧ），ゼラチン）を用いて１時間洗浄した。プレートを吸引し、そして、凍結乾燥機中、４℃で２４時間乾燥させた。プレートを、乾燥剤と一緒に小袋に入れた。

【０１３４】

抗原／抗体の組み合わせイムノアッセイを実行するために、１００μＬの増強溶解緩衝液（１％　Ｎ－ラウリルサルコシン、０．６５Ｍ　ＮａＣｌ、５０ｍｇ／ｍＬ　マウスＩｇＧ試験等級（Ｓｉｇｍａ，Ｓｔ．Ｌｏｕｉｓ，ＭＯ）、１％　スルフヒドリル改変ＢＳＡ（Ｂａｙｅｒ）、０．１％　カゼイン）を、このプレートに添加した。次いで、１００ｍＬのサンプルを、添加した。これを、振盪器上、４０℃で１時間インキュベートした。プレートを、Ｏｒｔｈｏ　Ｐｌａｔｅ　Ｗａｓｈｅｒ上、１×ＰＢＳ、０．１％　Ｔｗｅｅｎ－２０を用いて６回洗浄した。２００ｍＬの結合溶液（２５０ｎｇ／アッセイ　ＳＯＤ－ｃ３３ｃ抗原を有する１：７５希釈ｃ１１－１４－ＨＲＰ、ならびにＳＯＤ抽出物を含まないＨＣＶ３．０サンプル希釈物中の１：５０００希釈マウス抗ＳＯＤ－ＨＲＰ（ＯＲＴＨＯ　ＨＣＶ　バージョン　３．０　ＥＬＩＳＡ試験システム（Ｏｒｔｈｏ　Ｃｌｉｎｉｃａｌ　Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ　Ｊｅｒｓｅｙ）（全て、添加の３０分前に調製した）。この溶液を、振盪しながら４０℃で４５分間インキュベートした。これを、上記のように６回洗浄し、そして２００ｍＬの基質溶液（１ＯＰＤ錠剤／１０ｍＬ）を、添加した。ＯＰＤ錠剤は、西洋ワサビペルオキシダーゼ反応の色素発生のためのｏ－フェニレンジアミンジハイドロクロライドおよび過酸化水素を含み、そしてＳｉｇｍａ，Ｓｔ．Ｌｏｕｉｓ，ＭＯから市販されている。これを、３０分間、１５～３０℃で暗闇中インキュベートした。この反応を、５０ｍＬ　４Ｎ　H_2SO_4の添加によって停止させ、そしてプレートを、コントロールとしての６９０ｎｍでの吸光度に対して、４９２ｎｍで読み取った。

【０１３５】

（Ｄ．結果）

種々のアッセイの結果を、表５および表６に示し、これは示したようにＨＣＶ感染に曝露された血液サンプルで実施した２つの別々の実験を示す。斜線領域は、ウイルスの検出を示す。以下に示されるように、Ｃｈｉｒｏｎの組み合わせ抗原／抗体アッセイは、全てのサンプル中でセロコンバージョンを検出し、一方全ての他の抗体および抗原ベースのアッセイは、少なくとも１つのサンプルにおいてセロコンバージョンを検出することに失敗した。特に、いずれの抗体ベースのアッセイも、少なくとも１８日目までセロコンバージョンを検出しなかった（表５）。表６は、いずれの抗体ベースのアッセイも、ＨＣＶ感染の存在を２２日目で検出しなかったことを示す。さらに、Ｏｒｔｈｏ抗原ベースのアッセイは、８５日目からセロコンバージョンの検出に失敗した。

【０１３６】

従って、上記の結果に基づいて、新規の組み合わせ抗体／抗原アッセイが、他の従来の抗体および抗原ベースのアッセイを用いて得られる偽陰性数を減少させることは明らかである。

【０１３７】

【表５】

表　5
HCV セロコンバージョン

日数	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	$>5 \times 10^5$	9.25	18.6	2.8
4	0.1	0.0	$>5 \times 10^5$	9.29	19.0	3.1
7	0.1	0.0	$>5 \times 10^5$	9.52	22.3	1.5
13	0.3	0.1	$>5 \times 10^5$	9.59	26.2	1.7
18	1.3	0.4	$>5 \times 10^5$	9.70	15.9	1.2
21	2.2	1.0	$>5 \times 10^5$	9.39	11.3	1.5
164	4.2	4.4	4×10^4	9.28	0.11	2.5

【０１３８】

【表６】

表　6
HCV セロコンバージョン

日数	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	BLD		0.11	0.5
13	0.1	0.0	$>5 \times 10^5$		44.0	3.0
20	0.1	0.0	$>5 \times 10^5$		24.2	1.3
22	0.3	0.0	$>5 \times 10^5$		29.2	1.6
85	5.4	4.7	BQR		0.06	1.1
131	4.3	4.7	BQR		0.09	1.0
135	4.6	4.7	3×10^3		0.09	1.2
138	5.5	4.7	BLD		0.08	1.2
146	5.9	4.7	BLD		0.11	2.1
152	5.2	4.7	BQR		0.07	1.8

（実施例２）

（ＴｈｒからＰｒｏおよびＳｅｒからＩｌｅへの置換を伴うＮＳ３／４ａコンホメーショ

ンエピトープの産生）
ＮＳ３／４ａのコンホメーションエピトープを以下の通りに得た。このエピトープは、図４Ａ～４Ｄで指定した配列を有し、ネイティブな配列とは４０３位（ＨＣＶ－１全長配列のアミノ酸１４２８）および４０４位（ＨＣＶ－１全長配列のアミノ酸１４２９）で異なる。特に、ネイティブな配列の１４２８位で通常生じるＴｈｒはＰｒｏへと変異しており、そしてネイティブな配列の１４２９位で生じるＳｅｒは、Ｉｌｅへと変異している。
【０１３９】
特に、用いられる酵母発現ベクターは、上記のｐＢＳ２４．１であった。このイムノアッセイにおいて用いられる代表的なＮＳ３／４ａエピトープをコードするプラスミドｐｄ．ｈｃｖ１ａ．ｎｓ３ｎｓ４ａＰＩを、以下の通りに作製した。二工程手順を用いた。最初に、以下のＤＮＡ片を一緒に連結した：（ａ）５’ＨｉｎｄＩＩＩクローニング部位、続いて配列ＡＣＡＡＡＡＣＡＡＡ、イニシエーターＡＴＧ、およびアミノ酸１０２７で始まりアミノ酸１０４６のＢｇｌＩ部位へと続くＨＣＶ１ａについてのコドンを提供する合成オリゴヌクレオチド；（ｂ）ｐＡｃＨＬＴｎｓ３ｎｓ４ａＰＩ由来の６８３ｂｐのＢｇｌＩ－ＣｌａＩ制限フラグメント（アミノ酸１０４６～１２７４をコードする）；ならびに（ｃ）ＨｉｎｄＩＩＩおよびＣｌａＩで消化し、脱リン酸化し、そしてゲル精製したｐＳＰ７２ベクター（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ＷＩ，ＧｅｎＢａｎｋ／ＥＭＢＬ登録番号Ｘ６５３３２）。プラスミドｐＡｃＨＬＴｎｓ３ｎｓ４ａＰＩを、ＢＤ　Ｐｈａｒｍｉｎｇｅｎ（Ｓａｎ　Ｄｉｅｇｏ，ＣＡ）から市販されているバキュロウイルス発現ベクターであるｐＡｃＨＬＴから誘導した。特に、ｐＡｃＨＬＴ　ＥｃｏＲＩ－ＰｓｔＩベクターならびに以下のフラグメントを調製した：ＨＣＶ－１ゲノムのアミノ酸１０２７～１３３６に対応する９３５ｂｐのＥｃｏＲＩ－ＡｌｗｎＩ；ＨＣＶ－１ゲノムのアミノ酸１３３６～１４１９に対応する２４７ｂｐのＡｌｗｎＩ－ＳａｃＩＩ；ＨＣＶ－１ゲノムのアミノ酸１４４９～１５０９に対応する１７５ｂｐのＨｉｎｆＩ－ＢｇｌＩ；ＨＣＶ－１ゲノムのアミノ酸１５１０～１７１１に対応する６１９ｂｐのＢｇｌＩ－ＰｓｔＩ＋転写終結コドン。ＨＣＶ－１ゲノムのアミノ酸１４２０～１４４８に対応し、ＰＩ変異（Ｐｒｏへと変異させたＴｈｒ－１４２８、Ｉｌｅへと変異させたＳｅｒ－１４２９）を含む、合成によって作製した９１ｂｐのＳａｃＩＩ－ＨｉｎｆＩフラグメントを上記の１７５ｂｐのＨｉｎｆＩ－ＢｇｌＩフラグメントおよび６１９ｂｐのＢｇｌＩ－ＰｓｔＩフラグメントと連結し、そしてＳａｃＩＩおよびＰｓｔＩで消化したｐＧＥＭ－５Ｚｆ（＋）ベクター中にサブクローン化した。ｐＧＥＭ－５Ｚｆ（＋）は、市販のＥ．ｃｏｌｉベクター（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ＷＩ，ＧｅｎＢａｎｋ／ＥＭＢＬ登録番号Ｘ６５３０８）である。コンピテントなＨＢ　１０１細胞の形質転換、個々のクローンのミニスクリーニング分析および配列確認後、ｐＧＥＭ５．ＰＩクローン２由来の８８５ｂｐのＳａｃＩＩ－ＰｓｔＩフラグメントをゲル精製した。このフラグメントを、上記のＥｃｏＲＩ－ＡｌｗｎＩ　９３５ｂｐフラグメント、ＡｌｗｎＩ－ＳａｃＩＩ　２４７ｂｐフラグメントおよびｐＡｃＨＬＴ　ＥｃｏＲＩ－ＰｓｔＩベクターと連結した。得られた構築物を、ｐＡｃＨＬＴｎｓ３ｎｓ４ａＰＩと命名した。
【０１４０】
上記の連結混合物をＨＢ１０１コンピテント細胞中に形質転換し、そして１００μｇ／ｍｌアンピシリンを含有するＬｕｒｉａ寒天プレートにプレーティングした。個々のクローンのミニプレップ分析によって推定のポジティブを同定し、そのうちの２つを増幅した。ｐＳＰ７２　１ａＨＣのクローン番号１およびクローン番号２についてのプラスミドＤＮＡを、Ｑｉａｇｅｎ　Ｍａｘｉｐｒｅｐキットを用いて調製し、そして配列決定した。
【０１４１】
次いで、以下のフラグメントを一緒に連結した：（ａ）ｐＳＰ７２１ａＨＣ　＃１由来の７６１ｂｐのＨｉｎｄＩＩＩ－ＣｌａＩフラグメント（ｐＳＰ７２．１ａＨＣを、以下を一緒に連結することによって作製した：ＨｉｎｄＩＩＩおよびＣｌａＩで消化したｐＳＰ７２、５’ＨｉｎｄＩＩＩクローニング部位を提供する合成オリゴヌクレオチド、続いて配列ＡＣＡＡＡＡＣＡＡＡ、開始コドンＡＴＧおよびアミノ酸１０２７で始まり、アミノ

酸１０４６でのＢｇｌＩＩ部位へと続く、ＨＣＶ１ａについてのコドン、ならびにｐＡｃＨＬＴｎｓ３ｎｓ４ａＰＩ由来の６８３ｂｐのＢｇｌＩＩ－ＣｌａＩ制限フラグメント（アミノ酸１０４６～１２７４をコードする）；（ｂ）酵母ハイブリッドプロモーターＡＤＨ２／ＧＡＰＤＨについての１３５３ｂｐのＢａｍＨＩ－ＨｉｎｄＩＩＩフラグメント；（ｃ）ｐＡｃＨＬＴｎｓ３ｎｓ４ａＰＩ由来の１３２０ｂｐのＣｌａＩ－ＳａｌＩフラグメント（Ｔｈｒ　１４２８がＰｒｏへと変異し、そしてＳｅｒ　１４２９がＩｌｅへと変異したＨＣＶ１ａアミノ酸１０４６～１７１１をコードする）；ならびに（ｄ）ＢａｍＨＩおよびＳａｌＩで消化し、脱リン酸化し、そしてゲル精製したｐＢＳ２４．１酵母発現ベクター。連結混合物をコンピテントなＨＢ１０１中へと形質転換し、そして１００μｇ／ｍｌアンピシリンを含有するＬｕｒｉａ寒天プレートにプレーティングした。個々のコロニーのミニプレップ分析によって、予想された３４４６ｂｐのＢａｍＨＩ－ＳａｌＩ挿入物（ＡＤＨ２／ＧＡＰＤＨプロモーター、イニシエーターコドンＡＴＧおよびアミノ酸１０２７～１７１１のＨＣＶ１ａ　ＮＳ３／４ａ（図４Ａ～図４Ｄのアミノ酸１～６８６として示す）（Ｔｈｒ　１４２８（図４Ａ～４Ｄのアミノ酸位置４０３）がＰｒｏへと変異し、そしてＳｅｒ　１４２９（図４Ａ～４Ｄのアミノ酸位置４０４）がＩｌｅへと変異している）から構成される）を有するクローンを同定した。この構築物を、ｐｄ．ＨＣＶ１ａ．ｎｓ３ｎｓ４ａＰＩと命名した（図５を参照のこと）。

【０１４２】

Ｓ．ｃｅｒｅｖｉｓｉａｅ株ＡＤ３をｐｄ．ＨＣＶ１ａ．ｎｓ３ｎｓ４ａＰＩで形質転換し、そして１つの形質転換体を、培地中のグルコースの枯渇後の発現についてチェックした。組換えタンパク質は、クマシーブルー染色によって検出し、そしてＮＳ３のヘリカーゼドメインに対するポリクローナル抗体を用いて免疫ブロット分析によって確認したところ、酵母において高いレベルで発現された。

【０１４３】

（実施例３）

（ＮＳ３／４ａコンホメーションエピトープの精製）

ＮＳ３／４ａコンホメーションエピトープを以下の通りに精製した。ＮＳ３／４ａエピトープを発現する、上記由来のＳ．ｃｅｒｅｖｉｓｉａｅ細胞を、上記の通りに収集した。この細胞を溶解緩衝液（５０ｍＭ　Ｔｒｉｓ（ｐＨ８．０）、１５０ｍＭ　ＮａＣｌ、１ｍＭ　ＥＤＴＡ、１ｍＭ　ＰＭＳＦ、０．１μＭペプスタチン、１μＭロイペプチン）中に懸濁し、そして１：１：１の細胞：緩衝液：０．５ｍｍガラスビーズの比でガラスビーズを用いて、Ｄｙｎｏ－Ｍｉｌｌ（Ｗａｂ　Ｗｉｌｌｙ　Ａ．Ｂａｃｈｏｆｏｎ，Ｂａｓｅｌ，Ｓｗｉｔｚｅｒｌａｎｄ）またはそれに相当する装置において溶解した。溶解産物を４℃で３０１００×ｇで３０分間遠心分離し、そして不溶タンパク質画分を含むペレットを洗浄緩衝液（６ｍｌ／ｇ出発細胞ペレット重量）に添加し、そして室温で１５分間揺り動かした。洗浄緩衝液は、５０ｍＭ　$NaPO_4$（ｐＨ８．０）、０．３Ｍ　ＮａＣｌ、５ｍＭ　β－メルカプトエタノール、１０％グリセロール、０．０５％オクチルグルコシド、１ｍＭ　ＥＤＴＡ、１ｍＭ　ＰＭＳＦ、０．１μＭペプスタチン、１μＭロイペプチンからなっていた。細胞破片を、３０１００×ｇで３０分間、４℃での遠心分離によって取り出した。上清を捨て、そしてペレットを保持した。

【０１４４】

タンパク質を、このペレットから以下の通りに抽出した。６ｍｌ／ｇの抽出緩衝液を添加し、そして室温で１５分間揺り動かした。この抽出緩衝液は、５０ｍＭ　Ｔｒｉｓ（ｐＨ８．０）、１Ｍ　ＮａＣｌ、５ｍＭ　β－メルカプトエタノール、１０％グリセロール、１ｍＭ　ＥＤＴＡ、１ｍＭ　ＰＭＳＦ、０．１μＭペプスタチン、１μＭロイペプチンからなっていた。これを、３０１００×ｇで３０分間、４℃で遠心分離した。上清を保持し、そして以下の式を用いて１７．５％になるように硫酸アンモニウムを添加した：上清の容積（ｍｌ）×ｘ％硫酸アンモニウム／（１－ｘ％硫酸アンモニウム）＝上清へと添加される４．１Ｍ飽和硫酸アンモニウムのｍｌ。氷上で攪拌しながら硫酸アンモニウムを滴下し、そして溶液を氷上で１０分間攪拌した。溶液を１７７００×ｇで３０分間、４℃で遠

心分離し、そしてペレットを保持し、そして２℃～８℃で４８時間まで保存した。
【０１４５】
ペレットを再懸濁し、そしてポリＵカラム（Ｐｏｌｙ　Ｕ　Ｓｅｐｈａｒｏｓｅ　４Ｂ，Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ）に４℃で以下の通りに流した。ペレットを、ペレットの重量１グラムあたり６ｍｌのポリＵ平衡化緩衝液中に再懸濁した。この平衡化緩衝液は、２５ｍＭ　ＨＥＰＥＳ（ｐＨ８．０）、２００ｍＭ　ＮａＣｌ、５ｍＭ　ＤＴＴ（新たに添加した）、１０％グリセロール、１．２オクチルグルコシドからなっていた。この溶液を４℃で１５分間で揺り動かし、そして３１０００×ｇで３０分間、４℃で遠心分離した。
【０１４６】
ポリＵカラム（１グラムの出発ペレット重量あたり１ｍｌ樹脂）を調製した。線形流速は６０ｃｍ／時間であり、そしてパッキング流速は６０ｃｍ／時間の１３３％であった。このカラムを平衡化緩衝液で平衡化し、そして再懸濁した硫酸アンモニウムペレットの上清を、この平衡化したカラムにローディングした。このカラムを、平衡化緩衝液でベースラインになるまで洗浄し、そしてタンパク質を、以下のポリＵ溶出緩衝液中で１段階溶出で溶出させた：２５ｍＭ　ＨＥＰＥＳ（ｐＨ８．０）、１Ｍ　ＮａＣｌ、５ｍＭ　ＤＴＴ（新たに添加した）、１０％グリセロール、１．２オクチルグルコシド。カラムの溶出物をＳＤＳ－ＰＡＧＥ（クマシー染色した）で泳動し、そしてアリコートを凍結して－８０℃で保存した。ＮＳ３／４ａエピトープの存在を、ＮＳ３プロテアーゼドメインに対するポリクローナル抗体および５－１－１エピトープ（ＨＣＶ　４ａ）に対するモノクローナル抗体を用いたウェスタンブロットによって確認した。
【０１４７】
さらに、プロテアーゼ酵素活性を、以下の通りにして精製の間、モニタリングした。ＮＳ４Ａペプチド（ＫＫＧＳＶＶＩＶＧＲＩＶＬＳＧＫＰＡＩＩＰＫＫ）およびＮＳ３／４ａコンホメーションエピトープ含有サンプルを９０μｌの反応緩衝液（２５ｍＭ　Ｔｒｉｓ（ｐＨ７．５）、０．１５Ｍ　ＮａＣｌ、０．５ｍＭ　ＥＤＴＡ、１０％グリセロール、０．０５　ｎ－ドデシルＢ－Ｄ－マルトシド、５ｍＭ　ＤＴＴ）中に希釈し、そして室温で３０分間混合させた。９０μｌの混合物をマイクロタイタープレート（Ｃｏｓｔａｒ，Ｉｎｃ．，Ｃｏｒｎｉｎｇ，ＮＹ）に添加し、そして１０μｌのＨＣＶ基質（ＡｎａＳｐｅｃ，Ｉｎｃ．，Ｓａｎ　Ｊｏｓｅ　ＣＡ）を添加した。プレートを混合し、そしてＦｌｕｏｓｔａｒプレートリーダーで読み取った。結果を、１分間あたりの相対蛍光単位（ＲＦＵ）で表した。
【０１４８】
これらの方法を用いて、１Ｍ　ＮａＣｌ抽出物の産物は３．７ＲＦＵ／分の活性を含んでおり、硫酸アンモニウム沈澱物は７．５ＲＦＵ／分の活性を有し、そしてポリＵ精製の産物は１８．５ＲＦＵ／分の活性を有していた。
【０１４９】
（実施例４）
（競合研究）
以下の競合研究を、ＮＳ３／４ａコンホメーションエピトープが、他のＨＣＶ抗原と異なる抗体を検出するか否かを評価するために実施した。詳細には、ＮＳ３／４ａ抗原を、以下のようにしてｃ２００抗原と比較した。
【０１５０】
上記のように生成された０．５μｇおよび１．０μｇのＮＳ３／４ａまたはｃ２００（Ｈｅｐａｔｏｌｏｇｙ（１９９２）１５：１９－２５、ＯＲＴＨＯ　ＨＣＶ　Ｖｅｒｓｉｏｎ　３．０　ＥＬＩＳＡ　Ｔｅｓｔ　Ｓｙｓｔｅｍ，Ｏｒｔｈｏ－Ｃｌｉｎｉｃａｌ　Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ　Ｊｅｒｓｅｙにおいて利用可能）を、総量２２０μｌ（１×ＰＢＳ）で２０μｌのサンプルＰＨＶ９１４－５（感染個体の血液から獲得された初期セロコンバージョンの採血）と混合した。この混合物を、マイクロウェル中で３７℃で１時間インキュベートした。次いでこの混合物を、ＮＳ３／４ａコーテ

ィングしたプレートに移し、そして３７℃で１時間インキュベートした。プレートを洗浄し、そして以下のようにしてアッセイした。

【０１５１】

１μｇのｃ２００抗原を、総量約２２０μｌ中の１０μｌのサンプルＰＨＶ９１４－５に添加した。この混合物を、マイクロウェル中で３７℃で１時間インキュベートし、そして２００μｌをＮＳ３／４ａコーティングしたプレートに移し（１００ｎｇ／アッセイ）、そして３７℃で１時間インキュベートした。プレートを、１×ＰＢＳ、０．１％　Ｔｗｅｅｎ－２０を用いて５回洗浄した。２００μｌの結合溶液（上記）を添加し、そしてプレートをインキュベートし、そしてアッセイした。ＰＨＶ９１４－５および１×ＰＢＳからなるコントロール（抗原を含まない）もまた上記の通り処理した。

【０１５２】

結果を表７に示す。４列目に示される％阻害結果は、３列目－｛（２列目／３列目）×１００｝として算出する。見られ得る通り、これらのデータは、ＮＳ３４ａが、初期セロコンバージョン抗体により中和され、そしてｃ２００は中和されないことを示す。ＰＨＶ９１４－５　ｃ３３ｃ初期セロコンバージョンパネルのメンバーにおける抗体が、プレート上にコーティングされたＮＳ３４ａと反応する場合、強いシグナルを達成した。ｃ２００抗原は、これらの抗体により中和されなかった。このことは、表７の上パネルに示される。ＮＳ３４ａをＰＨＶ９１４－５サンプルと混合した場合、それは中和され、従って、マイクロプレート上にコーティングされたＮＳ３４ａと反応する抗体は、サンプル中に存在しなかった。これらのデータは、ＮＳ３４ａが、ｃ２００により検出される抗体とは異なるクラスの抗体を検出し得ることを示す。

【０１５３】

【表７】

c200抗原と比較した、初期c33cセロコンバージョンパネルにおけるNS34a抗原検出の差を示す競合研究

	c200		PHV914-5			コントロール 1×PBS		% 阻害			
			s			s					
	1ug		1.450			1.645		12			
	1ug		1.545			1.687		8			
	0.5ug		1.557			1.913		19			
	0.5ug		1.719			1.804		5			
	NS3/4a		PHV914-5								
			s			s					
	1ug		0.054			1.599		97			
	1ug		0.037			1.677		98			
	0.5ug		0.066			1.672		96			
	0.5ug		NA			1.524		NA			

表 7

（実施例５）

（ＮＳ３／４ａコンホメーションエピトープの安定性の研究）

アッセイの性能に対するＮＳ３／４ａエピトープの安定性の役割を評価するために、以下の研究を実施して、室温での時間に対するＮＳ３／４ａの免疫反応性を決定した。小アリコートのストックＮＳ３／４ａを、表８に示される間隔で、室温に静置し、次いで凍結させた。全てのバイアルを同時にコーティングし、そして２つの初期ＮＳ３セロコンバージョンパネルに対して試験した。

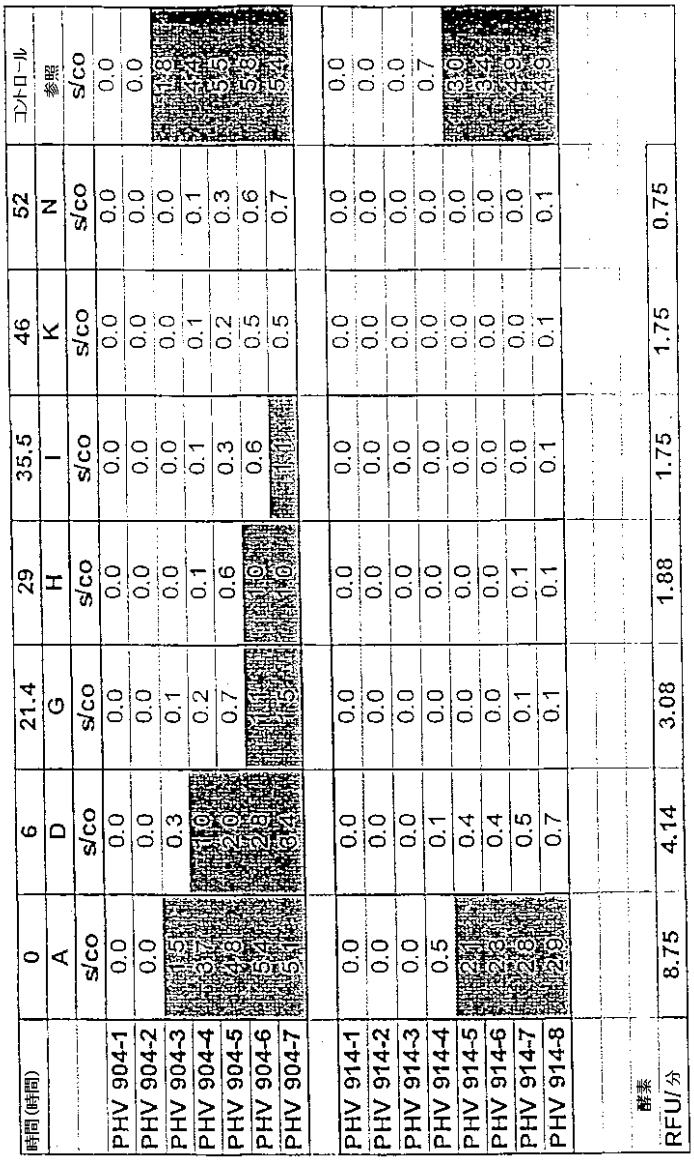
【０１５４】

表８に見られる通り、ＮＳ３／４ａストックは安定ではなく、そして時間と共に免疫反応性が減少する。さらに、ＮＳ３／４ａのコンホメーションを維持することは、免疫反応性のために必須である。

【０１５５】

さらなる安定性の研究を以下の通り実施した。標準的な手順を用いてＮＳ３／４ａに対して作製された２つのコンホメーションモノクローナル抗体を、抗ＨＣＶ初期セロコンバージョンパネルと置き換えた。ストックＮＳ３／４ａバイアルを、室温にて３、６、および２４時間間隔で保存した。凍結バイアル由来のＮＳ３／４ａを９０ｎｇ／ｍｌでコーティングし、そして上記の手順を用いてアッセイした。結果は、２つのモノクローナル抗体が実際にコンホメーション抗体であり、そしてそれらの反応性は室温でのストックＮＳ３／４ａ抗原の操作に対して感受性であったことを示唆した。ポジティブコントロールのモノクローナル抗体の反応性は変化しなかった。

【０１５６】
【表８】



時間（時間）	0		6		21.4		29		35.5		46		52		コントロール参照	
	A		D		G		H		I		K		N			
	s/co		s/co		s/co		s/co		s/co		s/co		s/co		s/co	
PHV 904-1	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 904-2	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 904-3	1.5		0.3		0.1		0.0		0.1		0.1		0.0		1.9	
PHV 904-4	3.7		1.0		0.2		0.1		0.1		0.1		0.1		4.4	
PHV 904-5	4.9		2.0		0.7		0.6		0.3		0.2		0.3		5.5	
PHV 904-6	5.4		2.9		1.1		1.0		0.6		0.5		0.6		5.8	
PHV 904-7	5.1		3.4		1.3		1.0		1.1		0.5		0.7		5.4	
PHV 914-1	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 914-2	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 914-3	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 914-4	0.5		0.1		0.0		0.0		0.0		0.0		0.0		0.7	
PHV 914-5	2.1		0.4		0.0		0.0		0.0		0.0		0.0		3.0	
PHV 914-6	2.3		0.4		0.0		0.0		0.0		0.0		0.0		3.4	
PHV 914-7	2.0		0.5		0.1		0.1		0.0		0.0		0.0		2.9	
PHV 914-8	2.9		0.7		0.1		0.1		0.1		0.1		0.1		4.9	
酵素																
RFU/分	8.75		4.14		3.08		1.88		1.75		1.75		0.75			

表８

（実施例６）
（変性ＮＳ３／４ａに対するＮＳ３／４ａコンホメーションエピトープの免疫反応性）
上記のように生成されたＮＳ３／４ａコンホメーションエピトープの免疫反応性を、ＮＳ３／４ａコンホメーションエピトープ調製物に最終濃度２％でＳＤＳを添加することにより変性させたＮＳ３／４ａと比較した。変性ＮＳ３／４ａおよび立体構造性のＮＳ３／４ａを、上記のようにマイクロタイタープレート上にコーティングした。ｃ２００抗原（Ｈｅｐａｔｏｌｏｇｙ（１９９２）１５：１９－２５、ＯＲＴＨＯ　ＨＣＶ　Ｖｅｒｓｉｏｎ　３．０　ＥＬＩＳＡ　Ｔｅｓｔ　Ｓｙｓｔｅｍ，Ｏｒｔｈｏ－Ｃｌｉｎｉｃａｌ　Ｄｉａｇｎｏｓｔｉｃｓ，Ｒａｒｉｔａｎ，Ｎｅｗ　Ｊｅｒｓｅｙにおいて利用可能）もまた、マイクロタイタープレート上にコーティングした。処方物中の還元剤（ＤＴＴ）および界面活性剤（ＳＤＳ）の存在に起因して非立体構造性であると推測されるｃ２００抗原を、比較として用いた。
【０１５７】
免疫反応性を、２つの初期ＨＣＶセロコンバージョンパネルＰＨＶ９０４およびＰＨＶ９１４（Ｂｏｓｔｏｎ　Ｂｉｏｍｅｄｉｃａ，Ｉｎｃ．，Ｗｅｓｔ　Ｂｒｉｄｇｅｗａｔｅｒ，ＭＡから市販されているヒト血液サンプル）に対して試験した。これらの結果を表９に示す。これらのデータは、変性または直鎖形態のＮＳ３／４ａ（およびｃ２００）が、

ＮＳ３／４ａコンホメーションエピトープと同じくらい早く初期セロコンバージョンパネルを検出しないことを示唆する。

【０１５８】

【表９】

	NS3/4a vs. 変性NS3/4a *ストックNS3／4aに対して2%SDSをスパイクした						
		NS3/4a	dNS3/4a*	c200	NS3/4a	dNS3/4a*	c200
		OD	OD	OD	s/co	s/co	s/co
HCV	PHV 904-1	0.012	0.012	0.009	0.02	0.02	0.01
セロコンバージョン	PHV 904-2	0.011	0.009	0.008	0.02	0.01	0.01
	PHV 904-3	1.124	0.071	0.045	1.80	0.11	0.07
	PHV 904-4	2.401	0.273	0.129	3.85	0.44	0.21
	PHV 904-5	3.022	0.793	0.347	4.85	1.28	0.57
	PHV 904-6	2.711	1.472	0.774	4.35	2.37	1.28
	PHV 904-7	3.294	1.860	0.943	5.28	3.00	1.55
	PHV 914-1	0.006	0.004	0.001	0.01	0.01	0.00
	PHV 914-2	0.005	0.004	0.002	0.01	0.01	0.00
	PHV 914-3	0.098	0.003	0.001	0.16	0.00	0.00
	PHV 914-4	1.118	0.006	0.004	1.79	0.01	0.01
	PHV 914-5	2.035	0.044	0.022	3.26	0.07	0.04
	PHV 914-6	2.092	0.074	0.025	3.35	0.12	0.04
	PHV 914-7	2.519	0.281	0.132	4.04	0.45	0.22
	PHV 914-8	2.746	0.907	0.500	4.40	1.46	0.82
	PHV 914-9	3.084	1.730	0.931	4.94	2.78	1.53
HCV 3.0	ネガティブコントロール	0.023	0.024	0.008			
コントロール	ネガティブコントロール	0.027	0.024	0.007			
	ネガティブコントロール	0.021	0.017	0.005			
	平均	0.024	0.022	0.007			
	カットオフ	0.624	0.622	0.607			
	ポジティブコントロール	1.239	0.903	0.575	1.99	1.45	0.95
	ポジティブコントロール	1.445	0.916	0.614	2.32	1.47	1.01

表 ９

コンホメーションエピトープの免疫反応性を、標準的な手順を用いて作製されたＮＳ３／４ａに対するモノクローナル抗体を用いても試験した。次いで、これらのモノクローナル抗体を、ＮＳ３／４ａおよび変性ＮＳ３／４ａならびにｃ２００抗原に対して、ＥＬＩＳＡフォーマットにおいて試験した。これらのデータは、抗ＮＳ３／４ａモノクローナル抗体が、表１０に示すセロコンバージョンパネルと同様の様式で、ＮＳ３／４ａおよび変性ＮＳ３／４ａと反応することを示す。この結果はまた、初期ｃ３３ｃセロコンバージョンパネルに対する反応性において類似するモノクローナル抗体が作製され得る場合、ＮＳ３／４ａが本質的に立体構造性であることのさらなる証拠を提供する。

【０１５９】

【表１０】

表 10

		プレート		
		NS3/4a	dNS3/4a	c200
モノクローナル		OD	OD	OD
4B9/E3	1:100	1.820	0.616	0.369
	1:1000	1.397	0.380	0.246
	1:10000	0.864	0.173	0.070
	1:20000	0.607	0.116	0.085
5B7/D7	1:100	2.885	0.898	0.436
	1:1000	2.866	0.541	0.267
	1:10000	1.672	0.215	0.086
	1:20000	1.053	0.124	0.059
1A8/H2	1:100	1.020	0.169	0.080
	1:1000	0.921	0.101	0.043
	1:10000	0.653	0.037	0.013
	1:20000	0.337	0.027	0.011

従って、新規のＨＣＶ検出アッセイを開示した。前述のことから、本発明の特定の実施形態が、例示の目的で本明細書中に記載されているが、本発明の意図および範囲から逸脱することなく種々の改変がなされ得ることが理解される。

【図面の簡単な説明】

【図１】

図１は、ＨＣＶゲノムの模式的表現であり、本発明のアッセイ試薬（タンパク質および抗体）が由来するポリタンパク質の種々の領域を示す。

【図２】

図２は、本発明の代表的な抗体／抗原組合せアッセイの模式図である。

【図３】

図３は、本発明のアッセイにおける使用のための代表的なＮＳ３／４ａコンホメーション抗原のアミノ酸配列を示す。１８２位の太文字のアラニンは、この位置に通常存在するネイティブなセリンに置換される。

【図４】

図４Ａ～４Ｄは、本発明のアッセイにおける使用のための別の代表的なＮＳ３／４ａコンホメーション抗原のＤＮＡおよび対応するアミノ酸配列を示す。図４Ａ～４Ｄの４０３位および４０４位のアミノ酸は、ＨＣＶ－１のネイティブなアミノ酸配列のＰｒｏのＴｈｒへの置換、およびＩｌｅのＳｅｒへの置換を表す。

【図５】

図５は、ｐｄ．ＨＣＶ１ａ．ｎｓ３ｎｓ４ａＰＩの構築の模式図である。

【図６】

図６は、ＭＥＦＡ　１２の模式的表現である。

【図７】

図７Ａ～７Ｆは、ＭＥＦＡ　１２のＤＮＡおよび対応するアミノ酸配列を示す。

【図８】
図８は、ＭＥＦＡ　１２を使用する、本発明の代表的なイムノアッセイの模式図である。

【図１】

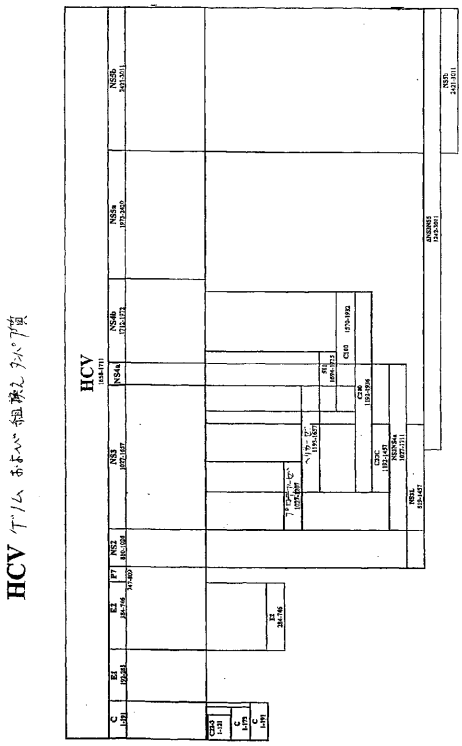


FIG. 1

【図２】

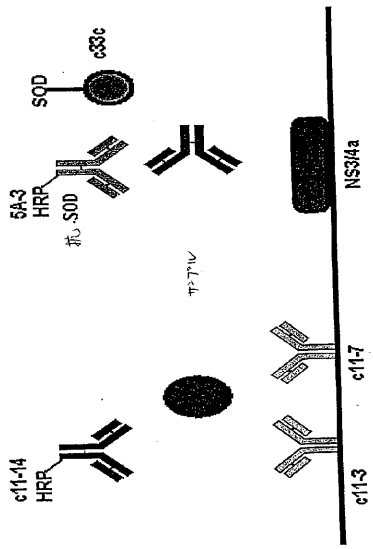


FIG. 2

【図５】

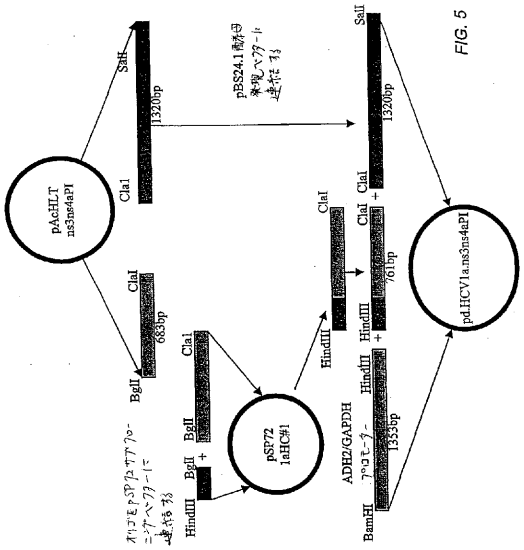


FIG. 5

【図６】

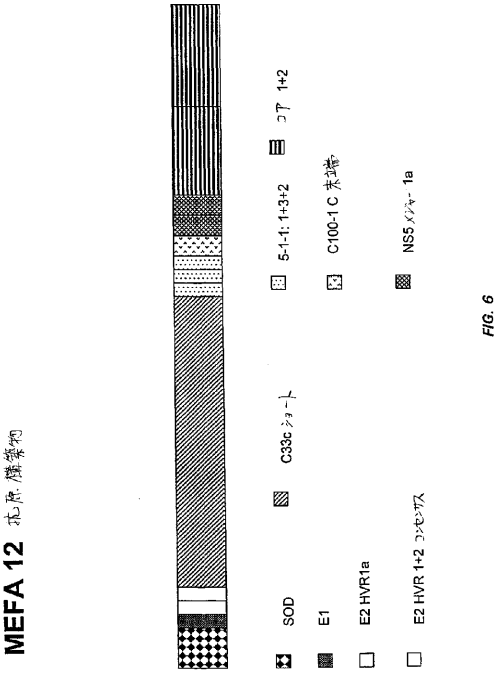


FIG. 6

【図８】

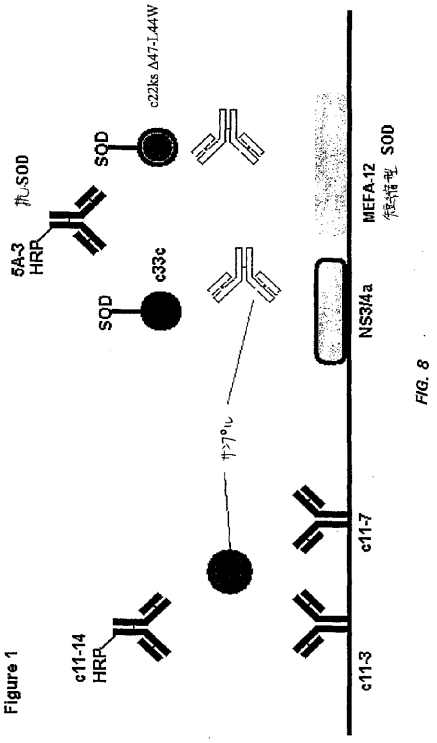


Figure 1

FIG. 8

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WO 01/96875 A2

(54) **Title:** HCV ANTIGEN/ANTIBODY COMBINATION ASSAY

(57) **Abstract:** An HCV core antigen and NS3/4a antibody combination assay that can detect both HCV antigens and antibodies present in a sample using a single solid matrix, is provided, as well as immunoassay solid supports for use in the assay.

HCV ANTIGEN/ANTIBODY COMBINATION ASSAY

Technical Field

The present invention pertains generally to viral diagnostics. In particular, the invention relates to an antigen/antibody combination assay for accurately diagnosing hepatitis C virus infection.

Background Of The Invention

Hepatitis C Virus (HCV) is the principal cause of parenteral non-A, non-B hepatitis (NANBH) which is transmitted largely through blood transfusion and sexual contact. The virus is present in 0.4 to 2.0% of blood donors. Chronic hepatitis develops in about 50% of infections and of these, approximately 20% of infected individuals develop liver cirrhosis which sometimes leads to hepatocellular carcinoma. Accordingly, the study and control of the disease is of medical importance.

HCV was first identified and characterized as a cause of NANBH by Houghten et al. The viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. HCV has a 9.5 kb positive-sense, single-stranded RNA genome and is a member of the Flaviridae family of viruses. At least six distinct, but related genotypes of HCV, based on phylogenetic analyses, have been identified (Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399). The virus encodes a single polyprotein having more than 3000 amino acid residues (Choo et al., *Science* (1989) 244:359-362; Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

In particular, as shown in Figure 1, several proteins are encoded by the HCV genome. The order and nomenclature of the cleavage products of the HCV polyprotein is as follows: NH_2-C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. Initial cleavage of the polyprotein is catalyzed by host proteases which liberate three structural proteins, the N-terminal nucleocapsid protein (termed "core") and two envelope glycoproteins, "E1" (also known as E) and "E2" (also known as E2/NS1), as well as nonstructural (NS) proteins that contain the viral enzymes. The NS regions are termed NS2, NS3, NS4 and NS5. NS2 is an integral membrane protein with proteolytic activity. NS2, either alone or in combination with NS3, cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease serves to process the remaining polyprotein. Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease. Subsequent NS3-mediated cleavages of the HCV polyprotein appear to involve recognition of polyprotein cleavage junctions by an NS3 molecule of another polypeptide. In these reactions, NS3 liberates an NS3 cofactor (NS4a), two proteins (NS4b and NS5a), and an RNA-dependent RNA polymerase (NS5b).

A number of general and specific polypeptides useful as immunological and diagnostic reagents for HCV, derived from the HCV polyprotein, have been described. See, e.g., Houghton et al., European Publication Nos. 318,216 and 388,232; Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Houghton et al., *Hepatology* (1991) 14:381-388; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778. These publications provide an extensive background on HCV generally, as well as on the manufacture and uses of HCV polypeptide immunological reagents.

Sensitive, specific methods for screening and identifying carriers of HCV and HCV-contaminated blood or blood products would provide an important advance in medicine. Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfused patients, and HCV has accounted for up to 90% of these cases. Patient care as well as the

prevention and transmission of HCV by blood and blood products or by close personal contact require reliable diagnostic and prognostic tools. Accordingly, several assays have been developed for the serodiagnosis of HCV infection. See, e.g., Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Choo et al., *Br. Med. Bull.* (1990) 46:423-441; Ebeling et al., *Lancet* (1990) 335:982-983; van der Poel et al., *Lancet* (1990) 335:558-560; van der Poel et al., *Lancet* (1991) 337:317-319; Chien, D.Y., International Publication No. WO 94/01778; Valenzuela et al., International Publication No. WO 97/44469; and Kashiwakuma et al., U.S. Patent No. 5,871,904.

A significant problem encountered with some serum-based assays is that there is a significant gap between infection and detection of the virus, often exceeding 80 days. This assay gap may create great risk for blood transfusion recipients. To overcome this problem, nucleic acid-based tests (NAT) that detect viral RNA directly, and HCV core antigen tests that assay viral antigen instead of antibody response, have been developed. See, e.g., Kashiwakuma et al., U.S. Patent No. 5,871,904; Beld et al., *Transfusion* (2000) 40:575-579.

However, there remains a need for sensitive, accurate diagnostic and prognostic tools in order to provide adequate patient care as well as to prevent transmission of HCV by blood and blood products or by close personal contact.

Summary of the Invention

The present invention is based in part, on the finding that HCV seroconversion antibodies are typically anti-core and anti-NS3 (helicase). Accordingly, the invention provides an HCV core antigen and NS3 antibody combination assay that can detect both HCV antigens and antibodies present in a sample using a single solid matrix.

Thus, in one embodiment, the subject invention is directed to an immunoassay solid support comprising at least one HCV anti-core antibody and at least one isolated HCV NS3/4a epitope bound thereto. The antibody and NS3/4a epitope can be any of the herein described molecules. Additionally, the solid support may include any of the multiple epitope fusion antigens described herein, such as the multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F.

In certain embodiments, the solid support comprises at least two HCV anti-core antibodies bound thereto. Moreover, the anti-core antibody may be a monoclonal antibody. Additionally, the NS3/4a epitope may be a conformational epitope, such as a conformational NS3/4a epitope comprising the amino acid sequence depicted in Figures 4A-4D.

In another embodiment, the invention is directed to an immunoassay solid support comprising at least two HCV anti-core monoclonal antibodies and at least one HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, bound thereto.

In still a further embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support as described above; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least one anti-core antibody and the NS3/4a epitope, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) an antigen that reacts with an HCV antibody from the biological sample reactive with the NS3/4a epitope; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the antigen of (ii); and (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample. The NS3/4a epitope may be a conformational epitope, such as a conformational epitope having the NS3/4a sequence depicted in Figures 4A-4D.

In yet another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support with at least two HCV anti-core antibodies bound thereto, as described above; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological

sample, to bind to the at least two anti-core antibodies and the NS3/4a epitope, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the hSOD amino acid sequence; and (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample. The NS3/4a epitope may be a conformational epitope, such as a conformational epitope having the NS3/4a sequence depicted in Figures 4A-4D.

In any of the above embodiments, the anti-core antibody may be directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence, and/or the detectably labeled HCV anti-core antibody may be directed against a C-terminal region of the HCV core antigen, such as amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence. Moreover, the antigen that reacts with an HCV antibody from the biological sample may be from the NS3 region, such as an epitope from the c33c region of the HCV polyprotein and can be fused with a human superoxide dismutase (hSOD) amino acid sequence. In this embodiment, the second detectably labeled antibody is reactive with the hSOD amino acid sequence.

In another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support including two HCV anti-core monoclonal antibodies and a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least two anti-core antibodies and the NS3/4a conformational epitope, respectively; adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled

antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with said hSOD amino acid sequence; detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

In certain embodiments, the at least two anti-core antibodies are directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein, and the detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen, such as against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

In another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support which includes a multiple epitope fusion antigen; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least one anti-core antibody, the NS3/4a epitope, and the multiple epitope fusion antigen; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) first and second antigens that react with an HCV antibody from the biological sample reactive with the NS3/4a epitope and the multiple epitope fusion antigen, respectively; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the antigens of (ii); (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

The anti-core antibody may be directed against an N-terminal region of the HCV core antigen and said first detectably labeled HCV anti-core antibody may be directed against a C-terminal region of the HCV core antigen, as described above. Moreover, the first antigen that reacts with an HCV antibody from the biological sample may comprise an epitope from the c33c region of the HCV polyprotein, and may be fused with an hSOD amino acid sequence. In this context, the second detectably labeled antibody is reactive with the hSOD amino acid sequence. Additionally, the second antigen that reacts with an HCV antibody from the biological sample may comprise an epitope from the c22 region of the HCV polyprotein, such as an epitope comprising amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence. The epitope may be fused with an hSOD amino acid sequence. If so, the second detectably labeled antibody is reactive with the hSOD amino acid sequence. The multiple epitope fusion antigen may comprise the amino acid sequence depicted in Figures 7A-7F.

In yet a further embodiment, the invention is directed to a method of detecting HCV infection in a biological sample, said method comprising: (a) providing an immunoassay solid support which comprises two HCV anti-core monoclonal antibodies, an HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, and a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, bound thereto; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least two anti-core antibodies, the NS3/4a conformational epitope, and the multiple epitope fusion antigen, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence and an epitope from the c22 region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody,

wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequences; (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

In this embodiment, the at least two anti-core antibodies may be directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein, and the detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen, such as against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence. Moreover, the epitope from the c22 region may comprise amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence.

In other embodiments, the invention is directed to immunodiagnostic test kits comprising the immunoassay solid support described above, and instructions for conducting the immunodiagnostic test.

In still further embodiments, the invention is directed to methods of producing an immunoassay solid support, comprising: (a) providing a solid support; and (b) binding at least one HCV anti-core antibody, such as one or two or more, and at least one isolated HCV NS3/4a epitope thereto, and optionally, a multiple epitope fusion antigen thereto. The anti-core antibodies, NS3/4a epitopes and multiple epitope fusion antigens are as described above.

In additional embodiments, the invention is directed to a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 80% sequence identity, such as 90% or more sequence identity, thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual. In certain embodiments, the multiple epitope fusion antigen consists of the amino acid sequence depicted in Figures 5A-5F.

In further embodiments, the invention is directed to a polynucleotide comprising a coding sequence for the multiple epitope fusion antigen above, a recombinant vectors comprising the polynucleotides, host cells transformed with the recombinant vectors, and methods of producing a recombinant multiple epitope fusion antigen comprising:

(a) providing a population of host cells as above; and (b) culturing the population of cells under conditions whereby the multiple epitope fusion antigen encoded by the coding sequence present in the recombinant vector is expressed.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the HCV genome, depicting the various regions of the polyprotein from which the present assay reagents (proteins and antibodies) are derived.

Figure 2 is a schematic drawing of a representative antibody/antigen combination assay under the invention.

Figure 3 depicts the amino acid sequence of a representative NS3/4a conformational antigen for use in the present assays. The bolded alanine at position 182 is substituted for the native serine normally present at this position.

Figures 4A through 4D depict the DNA and corresponding amino acid sequence of another representative NS3/4a conformational antigen for use in the present assays. The amino acids at positions 403 and 404 of Figures 4A through 4D represent substitutions of Pro for Thr, and Ile for Ser, of the native amino acid sequence of HCV-1.

Figure 5 is a diagram of the construction of pd.HCV1a.ns3ns4aPI.

Figure 6 is a diagrammatic representation of MEFA 12.

Figures 7A-7F depict the DNA and corresponding amino acid sequence of MEFA 12.

Figure 8 is a schematic drawing of a representative immunoassay under the invention, using MEFA 12.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the

literature. See, e.g., *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more antigens, and the like.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length

proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

An HCV polypeptide is a polypeptide, as defined above, derived from the HCV polyprotein. The polypeptide need not be physically derived from HCV, but may be synthetically or recombinantly produced. Moreover, the polypeptide may be derived from any of the various HCV strains, such as from strains 1, 2, 3 or 4 of HCV. A number of conserved and variable regions are known between these strains and, in general, the amino acid sequences of epitopes derived from these regions will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned. Thus, for example, the term "NS3/4a" polypeptide refers to native NS3/4a from any of the various HCV strains, as well as NS3/4a analogs, muteins and immunogenic fragments, as defined further below. The complete genotypes of many of these strains are known. See, e.g., U.S. Patent No. 6,150,087 and GenBank Accession Nos. AJ238800 and AJ238799.

The terms "analog" and "mutein" refer to biologically active derivatives of the reference molecule, or fragments of such derivatives, that retain desired activity, such as immunoreactivity in the assays described herein. In general, the term "analog" refers to compounds having a native polypeptide sequence and structure with one or more amino acid additions, substitutions (generally conservative in nature) and/or deletions, relative to the native molecule, so long as the modifications do not destroy immunogenic activity. The term "mutein" refers to peptides having one or more peptide mimics ("peptoids"), such as those described in International Publication No. WO 91/04282. Preferably, the analog or mutein has at least the same immunoactivity as the native molecule. Methods

for making polypeptide analogs and muteins are known in the art and are described further below.

Particularly preferred analogs include substitutions that are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. For example, the polypeptide of interest may include up to about 5-10 conservative or non-conservative amino acid substitutions, or even up to about 15-25 conservative or non-conservative amino acid substitutions, or any integer between 5-25, so long as the desired function of the molecule remains intact. One of skill in the art may readily determine regions of the molecule of interest that can tolerate change by reference to Hopp/Woods and Kyte-Doolittle plots, well known in the art.

By "fragment" is intended a polypeptide consisting of only a part of the intact full-length polypeptide sequence and structure. The fragment can include a C-terminal deletion and/or an N-terminal deletion of the native polypeptide. An "immunogenic fragment" of a particular HCV protein will generally include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably at least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between 5 amino acids and the full-length sequence, provided that the fragment in question retains immunoreactivity in the assays described herein. For example, preferred immunogenic fragments, include but are not limited to fragments of HCV core that comprise, e.g., amino acids 10-45, 10-53, 67-88, and 120-

130 of the polyprotein, epitope 5-1-1 (in the NS3 region of the viral genome) as well as defined epitopes derived from the E1, E2, c33c (NS3), c100 (NS4), NS3/4a and NS5 regions of the HCV polyprotein, as well as any of the other various epitopes identified from the HCV polyprotein. See, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; U.S. Patent Nos. 6,150,087 and 6,121,020.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, binds to an antibody generated in response to such sequence. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes from the HCV polyprotein. An epitope for use in the subject invention is not limited to a polypeptide having the exact sequence of the portion of the parent protein from which it is derived. Indeed, viral genomes are in a state of constant flux and contain several variable domains which exhibit relatively high degrees of variability between isolates. Thus the term "epitope" encompasses sequences identical to the native sequence, as well as modifications to the native sequence, such as deletions, additions and substitutions (generally conservative in nature).

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:178-182; Geysen et al. (1986) *Molec. Immunol.* 23:709-715.

Using such techniques, a number of epitopes of HCV have been identified. See, e.g., Chien et al., *Viral Hepatitis and Liver Disease* (1994) pp. 320-324, and further below. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols*, *supra*. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omiga version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

As used herein, the term "conformational epitope" refers to a portion of a full-length protein, or an analog or mutein thereof, having structural features native to the amino acid sequence encoding the epitope within the full-length natural protein. Native structural features include, but are not limited to, glycosylation and three dimensional structure. The length of the epitope defining sequence can be subject to wide variations as these epitopes are believed to be formed by the three-dimensional shape of the antigen (e.g., folding). Thus, amino acids defining the epitope can be relatively few in number, but widely dispersed along the length of the molecule (or even on different molecules in the case of dimers, etc.), being brought into correct epitope conformation via folding. The portions of the antigen between the residues defining the epitope may not be critical to the conformational structure of the epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g., cysteines involved in disulfide bonding, glycosylation sites, etc.).

Conformational epitopes present in the NS3/4a region are readily identified using methods discussed above. Moreover, the presence or absence of a conformational epitope in a given polypeptide can be readily determined through screening the antigen of interest with an antibody (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains

only linear epitopes (if any). In such screening using polyclonal antibodies, it may be advantageous to absorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest. Additionally, in the case of NS3/4a, a molecule which preserves the native conformation will also have protease and, optionally, helicase enzymatic activities. Such activities can be detected using enzymatic assays, as described further below.

Preferably, a conformational epitope is produced recombinantly and is expressed in a cell from which it is extractable under conditions which preserve its desired structural features, e.g. without denaturation of the epitope. Such cells include bacteria, yeast, insect, and mammalian cells. Expression and isolation of recombinant conformational epitopes from the HCV polyprotein are described in e.g., International Publication Nos. WO 96/04301, WO 94/01778, WO 95/33053, WO 92/08734. Alternatively, it is possible to express the antigens and further renature the protein after recovery. It is also understood that chemical synthesis may also provide conformational antigen mimitopes that cross-react with the "native" antigen's conformational epitope.

The term "multiple epitope fusion antigen" or "MEFA" as used herein intends a polypeptide in which multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or may be separated by intervening amino acid sequences. The fusion antigens may also contain sequences exogenous to the HCV polyprotein. Moreover, the HCV sequences present may be from multiple genotypes and/or isolates of HCV. Examples of particular MEFAs for use in the present immunoassays are detailed in, e.g., International Publication No. WO 97/44469, and are described further below.

An "antibody" intends a molecule that, through chemical or physical means, specifically binds to a polypeptide of interest. Thus, an HCV core antibody is a molecule that specifically binds to the HCV core protein. The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Patent No. 4,816,567); F(ab')$_2$ and F(ab)

fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar et al. (1972) *Proc Natl Acad Sci USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem* 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) *Proc Natl Acad Sci USA* 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. Thus, the term encompasses antibodies obtained from murine hybridomas, as well as human monoclonal antibodies obtained using human rather than murine hybridomas. See, e.g., Cote, et al. *Monclonal Antibodies and Cancer Therapy*, Alan R. Liss, 1985, p. 77.

A "recombinant" protein is a protein which retains the desired activity and which has been prepared by recombinant DNA techniques as described herein. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macro-molecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

By "equivalent antigenic determinant" is meant an antigenic determinant from different sub-species or strains of HCV, such as from strains 1, 2, or 3 of HCV. More specifically, epitopes are known, such as 5-1-1, and such epitopes vary between the strains 1, 2, and 3. Thus, the epitope 5-1-1 from the three different strains are equivalent antigenic determinants and thus are "copies" even though their sequences are not identical. In general the amino acid sequences of equivalent antigenic determinants will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned.

"Homology" refers to the percent similarity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence similarity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100.

Readily available computer programs can be used to aid in the analysis of similarity and identity, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence similarity and identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the

default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent similarity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Another method of establishing percent similarity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence similarity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the

case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper transcription factors, etc., are present. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence, as can transcribed introns, and the promoter sequence can still be considered "operably linked" to the coding sequence.

A "control element" refers to a polynucleotide sequence which aids in the expression of a coding sequence to which it is linked. The term includes promoters, transcription termination sequences, upstream regulatory domains, polyadenylation signals, untranslated regions, including 5'-UTRs and 3'-UTRs and when appropriate, leader sequences and enhancers, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A "promoter" as used herein is a DNA regulatory region capable of binding RNA polymerase in a host cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence

into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

"Expression cassette" or "expression construct" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette includes control elements, as described above, such as a promoter which is operably linked to (so as to direct transcription of) the sequence(s) or gene(s) of interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the expression cassette described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (e.g., a M13 origin of replication), at least one multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

"Transformation," as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

"Common solid support" intends a single solid matrix to which the HCV polypeptides used in the subject immunoassays are bound covalently or by noncovalent means such as hydrophobic adsorption.

"Immunologically reactive" means that the antigen in question will react specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

"Immune complex" intends the combination formed when an antibody binds to an epitope on an antigen.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, chromophores, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin, strepavidin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include, but are not limited to, horse radish peroxidase (HRP), fluorescein, FITC, rhodamine, dansyl, umbelliferone, dimethyl acridinium ester (DMAE), Texas red, luminol, NADPH and α-β-galactosidase.

II. Modes of Carrying out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

As noted above, the present invention is based on the discovery of novel diagnostic methods for accurately detecting early HCV infection. The methods rely on the identification and use of highly immunogenic HCV antibodies and antigens which are

present during the early stages of HCV seroconversion, thereby increasing detection accuracy and reducing the incidence of false results. The methods can be conveniently practiced in a single assay format.

More particularly, the assay is conducted on a solid support to which has been bound one or more HCV anti-core antibodies (directed against either the same or different HCV core epitopes) and an epitope derived from the NS3/4a region of the HCV polyprotein. Examples of particular anti-core antibodies useful in the present invention include, but are not limited to, antibody molecules such as monoclonal antibodies, directed against epitopes in the core region found between amino acids 10-53; amino acids 10-45; amino acids 67-88; amino acids 120-130, or antibodies directed against any of the core epitopes identified in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818.

The NS3/4a region of the HCV polyprotein has been described and the amino acid sequence and overall structure of the protein are disclosed in, e.g., Yao et al., *Structure* (November 1999) 7:1353-1363; Sali et al., *Biochem.* (1998) 37:3392-3401; and Bartenschlager, R., *J. Viral Hepat.* (1999) 6:165-181. See, also, Dasmahapatra et al., U.S. Patent No. 5,843,752. The subject immunoassays utilize at least one conformational epitope derived from the NS3/4a region that exists in the conformation as found in the naturally occurring HCV particle or its infective product, as evidenced by the preservation of protease and, optionally, helicase enzymatic activities normally displayed by the NS3/4a gene product and/or immunoreactivity of the antigen with antibodies in a biological sample from an HCV-infected subject, and a loss of the epitope's immunoreactivity upon denaturation of the antigen. For example, the conformational epitope can be disrupted by heating, changing the pH to extremely acid or basic, or by adding known organic denaturants, such as dithiothreitol (DTT) or an appropriate detergent. See, e.g., *Protein Purification Methods, a practical approach* (E.L.V. Harris

and S. Angal eds., IRL Press) and the denatured product compared to the product which is not treated as above.

Protease and helicase activity may be determined using standard enzyme assays well known in the art. For example, protease activity may be determined using assays well known in the art. See, e.g., Takeshita et al., *Anal. Biochem.* (1997) 247:242-246; Kakiuchi et al., *J. Biochem.* (1997) 122:749-755; Sali et al., *Biochemistry* (1998) 37:3392-3401; Cho et al., *J. Virol. Meth.* (1998) 72:109-115; Cerretani et al., *Anal. Biochem.* (1999) 266:192-197; Zhang et al., *Anal. Biochem.* (1999) 270:268-275; Kakiuchi et al., *J. Virol. Meth.* (1999) 80:77-84; Fowler et al., *J. Biomol. Screen.* (2000) 5:153-158; and Kim et al., *Anal. Biochem.* (2000) 284:42-48. A particularly convenient assay for testing protease activity is set forth in the examples below.

Similarly, helicase activity assays are well known in the art and helicase activity of an NS3/4a epitope may be determined using, for example, an ELISA assay, as described in, e.g., Hsu et al., *Biochem. Biophys. Res. Commun.* (1998) 253:594-599; a scintillation proximity assay system, as described in Kyono et al., *Anal. Biochem.* (1998) 257:120-126; high throughput screening assays as described in, e.g., Hicham et al., *Antiviral Res.* (2000) 46:181-193 and Kwong et al., *Methods Mol. Med.* (2000) 24:97-116; as well as by other assay methods known in the art. See, e.g., Khu et al., *J. Virol.* (2001) 75:205-214; Utama et al., *Virology* (2000) 273:316-324; Paolini et al., *J. Gen. Virol.* (2000) 81:1335-1345; Preugschat et al., *Biochemistry* (2000) 39:5174-5183; Preugschat et al., *Methods Mol. Med.* (1998) 19:353-364; and Hesson et al., *Biochemistry* (2000) 39:2619-2625.

The length of the antigen is sufficient to maintain an immunoreactive conformational epitope. Often, the polypeptide containing the antigen used will be almost full-length, however, the polypeptide may also be truncated to, for example, increase solubility or to improve secretion. Generally, the conformational epitope found in NS3/4a is expressed as a recombinant polypeptide in a cell and this polypeptide provides the epitope in a desired form, as described in detail below.

Representative amino acid sequences for NS3/4a polypeptides are shown in Figure 3 and Figures 4A through 4D. The bolded alanine occurring at position 182 of

Figure 3 is substituted for the native serine found at this position in order to prevent autocatalyisis of the molecule that might otherwise occur. The amino acid sequence shown at positions 2-686 of Figures 4A through 4D corresponds to amino acid positions 1027-1711 of HCV-1. An initiator codon (ATG) coding for Met, is shown as position 1. Additionally, the Thr normally occurring at position 1428 of HCV-1 (amino acid position 403 of Figure 4) is mutated to Pro, and the Ser normally occurring at position 1429 of HCV-1 (amino acid position 404 of Figure 4) is mutated to Ile. However, either the native sequence, with or without an N-terminal Met, the depicted analog, with or without the N-terminal Met, or other analogs and fragments can be used in the subject assays, so long as the epitope is produced using a method that retains or reinstates its native conformation such that protease activity, and optionally, helicase activity is retained. Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276, both describe analogs of NS3/4a.

The NS3 protease of NS3/4a is found at about positions 1027-1207, numbered relative to HCV-1, positions 2-182 of Figure 4. The structure of the NS3 protease and active site are known. See, e.g., De Francesco et al., *Antivir. Ther.* (1998) 3:99-109; Koch et al., *Biochemistry* (2001) 40:631-640. Changes to the native sequence that will normally be tolerated will be those outside of the active site of the molecule. Particularly, it is desirable to maintain amino acids 1- or 2-155 of Figure 4, with little or only conservative substitutions. Amino acids occurring beyond 155 will tolerate greater changes. Additionally, if fragments of the NS3/4a sequence found in Figure 4 are used, these fragments will generally include at least amino acids 1- or 2-155, preferably amino acids 1- or 2-175, and most preferably amino acids 1- or 2-182, with or without the N-terminal Met. The helicase domain is found at about positions 1193-1657 of HCV-1 (positions 207-632 of Figure 4). Thus, if helicase activity is desired, this portion of the molecule will be maintained with little or only conservative changes. One of skill in the art can readily determine other regions that will tolerate change based on the known structure of NS3/4a.

The solid support may also comprise other antigens. For example, multiple epitope fusion antigens (termed "MEFAs"), as described in International Publication No.

WO 97/44469, may be bound to the solid support for use in the subject assays. Such MEFAs include multiple epitopes derived from two or more of the various viral regions shown in Figure 1 and Table 1. In particular, as shown in Figure 1 and Table 1, An HCV polyprotein, upon cleavage, produces at least ten distinct products, in the order of NH_2-Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. The core polypeptide occurs at positions 1-191, numbered relative to HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, for the HCV-1 genome). This polypeptide is further processed to produce an HCV polypeptide with approximately amino acids 1-173. The envelope polypeptides, E1 and E2, occur at about positions 192-383 and 384-746, respectively. The P7 domain is found at about positions 747-809. NS2 is an integral membrane protein with proteolytic activity and is found at about positions 810-1026 of the polyprotein. NS2, either alone or in combination with NS3 (found at about positions 1027-1657), cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease, found at about positions 1027-1207, serves to process the remaining polyprotein. The helicase activity is found at about positions 1193-1657. Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease. Subsequent NS3-mediated cleavages of the HCV polyprotein appear to involve recognition of polyprotein cleavage junctions by an NS3 molecule of another polypeptide. In these reactions, NS3 liberates an NS3 cofactor (NS4a, found about positions 1658-1711), two proteins (NS4b found at about positions 1712-1972, and NS5a found at about positions 1973-2420), and an RNA-dependent RNA polymerase (NS5b found at about positions 2421-3011).

Table 1	
Domain	Approximate Boundaries*
C (core)	1-191
E1	192-383
E2	384-746
P7	747-809
NS2	810-1026
NS3	1027-1657
NS4a	1658-1711
NS4b	1712-1972
NS5a	1973-2420
NS5b	2421-3011

*Numbered relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455.

The multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. Thus, the linear order of the epitopes is different than their linear order in the genome in which they occur. The linear order of the sequences of the MEFAs for use herein is preferably arranged for optimum antigenicity. Preferably, the epitopes are from more than one HCV strain, thus providing the added ability to detect multiple strains of HCV in a single assay. Thus, the MEFAs for use herein may comprise various immunogenic regions derived from the polyprotein described above. Moreover, a protein resulting from a frameshift in the core region of the polyprotein, such as described in International Publication No. WO 99/63941, may be used in the MEFAs. If desired, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more of one or more epitopes derived from the HCV polyprotein may occur in the fusion protein.

For example, epitopes derived from, e.g., the hypervariable region of E2, such as a region spanning amino acids 384-410 or 390-410, can be included in the MEFA antigen. A particularly effective E2 epitope is one which includes a consensus sequence derived from this region, such as the consensus sequence Gly-Ser-Ala-Ala-Arg-Thr-Thr-Ser-Gly-Phe-Val-Ser-Leu-Phe-Ala-Pro-Gly-Ala-Lys-Gln-Asn, which represents a consensus sequence for amino acids 390-410 of the HCV type 1 genome. A representative E2 epitope present in a MEFA of the invention can comprise a hybrid epitope spanning amino acids 390-444. Such a hybrid E2 epitope can include a consensus sequence representing amino acids 390-410 fused to the native amino acid sequence for amino acids 411-444 of HCV E2.

Additionally, the antigens may be derived from various HCV strains. Multiple viral strains of HCV are known, and epitopes derived from any of these strains can be used in a fusion protein. It is well known that any given species of organism varies from one individual organism to another and further that a given organism such as a virus can have a number of different strains. For example, as explained above, HCV includes at least 6 genotypes. Each of these genotypes includes equivalent antigenic determinants. More specifically, each strain includes a number of antigenic determinants that are present on all strains of the virus but are slightly different from one viral strain to another. For example, HCV includes the antigenic determinant known as 5-1-1 (See, Figure 1). This particular antigenic determinant appears in three different forms on the three different viral strains of HCV. Accordingly, in a preferred embodiment of the invention all three forms of 5-1-1 appear on the multiple epitope fusion antigen used in the subject immunoassays. Similarly, equivalent antigenic determinants from the core region of different HCV strains may also be present. In general, equivalent antigenic determinants have a high degree of homology in terms of amino acid sequence which degree of homology is generally 30% or more, preferably 40% or more, when aligned. The multiple copy epitope of the present invention can also include multiple copies which are exact copies of the same epitope.

Representative MEFAs for use with the present assays are described in International Publication No. WO 97/44469. Additional representative MEFAs for use

herein include those termed MEFA 12, MEFA 13 and MEFA 13.1. It is to be understood that these MEFAs are merely representative and other epitopes derived from the HCV genome will also find use with the present assays and may be incorporated into these or other MEFAs.

The DNA sequence and corresponding amino acid sequence of MEFA 12 is shown in Figures 7A through 7F. The general structural formula for MEFA 12 is shown in Figure 6 and is as follows: hSOD-E1(type 1)-E2 HVR consensus(type 1a)-E2 HVR consensus(types 1 and 2)-c33c short(type 1)-5-1-1(type 1)-5-1-1(type 3)-5-1-1(type 2)-c100(type 1)-NS5(type 1)-NS5(type 1)-core(types 1+2)-core(types 1+2). This multiple copy epitope includes the following amino acid sequence, numbered relative to HCV-1 (the numbering of the amino acids set forth below follows the numbering designation provided in Choo, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, in which amino acid #1 is the first methionine encoded by the coding sequence of the core region): amino acids 1-69 of superoxide dismutase (SOD, used to enhance recombinant expression of the protein); amino acids 303 to 320 of the polyprotein from the E1 region; amino acids 390 to 410 of the polyprotein, representing a consensus sequence for the hypervariable region of HCV-1a E2; amino acids 384 to 414 of the polyprotein from region E2, representing a consensus sequence for the E2 hypervariable regions of HCV-1 and HCV-2; amino acids 1211-1457 of the HCV-1 polyprotein which define the helicase; three copies of an epitope from 5-1-1, amino acids 1689-1735, one from HCV-1, one from HCV-3 and one from HCV-2, which copies are equivalent antigenic determinants from the three different viral strains of HCV; HCV polypeptide C100 of HCV-1, amino acids 1901-1936 of the polyprotein; two exact copies of an epitope from the NS5 region of HCV-1, each with amino acids 2278 to 2313 of the HCV polyprotein; and two copies of three epitopes from the core region, two from HCV-1 and one from HCV-2, which copies are equivalent antigenic determinants represented by amino acids 9 to 53 and 64-88 of HCV-1 and 67-84 of HCV-2.

Table 2 shows the amino acid positions of the various epitopes in MEFA 12 with reference to Figures 7A through 7F herein. The numbering in the tables is relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455. MEFAs 13

and 13.1 also share the general formula specified above for MEFA 12, with modifications as indicated in Tables 3 and 4, respectively.

Table 2. MEFA 12

mefa aa#	5' end site	epitope	hcv aa#	strain
1-69*	*Nco*1	hSOD		
72-89	*Mlu*I	E1	303-320	1
92-112	*Hind*111	E2 HVR1a consensus	390-410	1
113-143		E2 HVR1+2 consensus	384-414	1, 2
146-392	*Spe*I	C33C short	1211-1457	1
395-441	*Sph*I	5-1-1	1689-1735	1
444-490	*Nru*I	5-1-1	1689-1735	3
493-539	*Cla*I	5-1-1	1689-1735	2
542-577	*Ava*I	C100	1901-1936	1
580-615	*Xba*I	NS5	2278-2313	1
618-653	*Bgl*II	NS5	2278-2313	1
654-741	*Nco*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2
742-829	*Bal*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The SOD protein is truncated so that so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

Table 3. MEFA 13

mefa aa#	5' end site	epitope	hcv aa#	strain
1-156	*Nco*1	mutated hSOD (aa 70-72, ALA)		
161-178	*Mlu*I	E1	303-320	1
181-201	*Hind*111	E2 HVR1a consensus	390-410	1
202-232		E2 HVR1+2 consensus	384-414	1, 2
235-451		C33C short	1211-1457	1
454-500	*Hind*III	5-1-1 PImut*	1689-1735	1
503-549	*Nru*I	5-1-1 PImut*	1689-1735	3
552-598	*Cla*I	5-1-1 PImut*	1689-1735	2
601-636	*Ava*I	C100	1901-1936	1
639-674	*Xba*I	NS5	2278-2313	1
677-712	*Bgl*II	NS5	2278-2313	1
713-800		core epitopes	9-53, R47L 64-88 67-84	1 1 2
801-888		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI. Additionally, the SOD protein is mutated so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

Table 4. MEFA 13.1

mefa aa#	5' end site	epitope	hcv aa#	strain
1-86	*Nco*I	mutated hSOD (aa 70-72, ALA)		
89-106	*Mlu*I	E1	303-320	1
109-129	*Hind*III	E2 HVR1a consensus	390-410	1
130-160		E2 HVR1+2 consensus	384-414	1, 2
163-379		C33C short	1211-1457	1
382-428	*Hind*III	5-1-1 PImut*	1689-1735	1
431-477	*Nru*I	5-1-1 PImut*	1689-1735	3
480-526	*Cla*I	5-1-1 PImut*	1689-1735	2
529-564	*Ava*I	C100	1901-1936	1
567-602	*Xba*I	NS5	2278-2313	1
605-640	*Bgl*II	NS5	2278-2313	1
641-728		core epitopes	9-53, R47L 64-88 67-84	1 1 2
729-816		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI. Additionally, the SOD protein is mutated so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

In one assay format, the sample is combined with the solid support, as described further below. If the sample is infected with HCV, core antigens, as well as HCV

antibodies to those epitopes present on the solid support, will bind to the solid support components. A detectably labeled anti-core antibody is then added. The labeled anti-core antibody is directed against a different epitope than the anti-core antibody that is bound to the solid support. This anti-core antibody binds the core antigen captured by the anti-core antibodies on the solid support.

An antigen that reacts with the captured HCV antibody from the biological sample, which captured sample HCV antibody is reactive with the NS3/4a epitope, is also added. This antigen is preferably an epitope derived from the NS3 region of the HCV polyprotein. This antigen binds the captured HCV antibody from the sample. A number of antigens including such epitopes are known, including, but not limited to antigens derived from the c33c and c100 regions, as well as fusion proteins comprising an NS3 epitope, such as c25. These and other NS3 epitopes are useful in the present assays and are known in the art and described in, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818.

A second labeled antibody, directed against the antigen described above, is added. This antibody can be directed against any epitope included in the antigen. For example, the antibody can be directed against the NS3 region present in the antigen. Alternatively, if the antigen above is expressed as a fusion protein, the second labeled antibody can be directed against the fusion partner. Additional antigens and antibodies can be added to the assay, particularly if the solid support includes a MEFA. These assay formats are explained further below.

A representative assay under the invention is depicted in Figure 2. As shown in the figure, the solid support includes two anti-core monoclonal antibodies, termed c11-3 and c11-7. These antibodies are directed against an epitope found in the N-terminal region of the core protein at amino acids 10-53, numbered relative to the HCV1 polyprotein sequence. The solid support also includes an epitope to NS3/4a. The biological sample is added to the solid support. HCV core antigen, as well as antibodies

directed against the NS3/4a epitope, both present in the sample, will bind the capture reagents on the solid support.

Horse radish peroxidase (HRP)-labeled anti-core monoclonal antibody c11-14, directed against a C-terminal region of the core found at amino acid positions 120-130, numbered relative to the HCV1 polyprotein sequence, is then added. A fusion protein, comprising a sequence from human SOD (hSOD) and an epitope from the c33c region is added, as is a second HRP-labeled antibody, directed against the SOD portion of the fusion protein. The SOD-c33c fusion will bind to the anti-NS3 antibody and the anti-SOD antibody will, in turn, bind the SOD-c33c fusion protein. Detection of the label indicates the presence of HCV infection.

Another representative assay under the invention is depicted in Figure 8. The antibody assay configuration is an antigen-antibody-antigen sandwich capture assay using both NS3/4a and MEFA 12. The solid support includes the two anti-core monoclonal antibodies described above, an epitope to NS3/4a, as well as a representative MEFA, MEFA 12, which includes a truncated version of human SOD. As with the assay above, the biological sample is added to the solid support. HCV core antigen, as well as antibodies directed against the NS3/4a epitope and epitopes of the MEFA, present in the sample, will bind the capture reagents on the solid support. Two antigens, one reactive with sample antibodies that bind NS3/4a (as described above) and one reactive with sample antibodies that bind MEFA 12, are added. In Figure 8, the antigen reactive with the MEFA 12/sample antibody complex is a fusion between an SOD molecule and c22ks Δ47-L44W. The c22ks antigen is from the core region and includes amino acids Lys_{10} to Ser_{99} of the polyprotein, as well as a deletion of Arg47 normally present and a substitution of Leu for Trp at position 44. The antibody detection conjugate is the second HRP-labeled monoclonal anti-SOD antibody, described above.

The above-described antigen/antibody combination assays are particularly advantageous as both the HCV core antigen and antibodies to NS3/4a and/or core may be detected by the same support in the same assay. Moreover, as described above, additional HCV epitopes, such as SOD-fused to c100, 5-1-1, NS5 antigens, as well as a protein resulting from a frameshift in the core region of the polyprotein, such as

described in International Publication No. WO 99/63941, may be used in the combination cocktail to cover other non-structural epitopes of HCV.

In order to further an understanding of the invention, a more detailed discussion is provided below regarding production of antibodies for use in the subject immunoassays; production of polypeptides for use in the immunoassays; and methods of conducting the immunoassays.

Production of Antibodies for use in the HCV Immunoassays

As explained above, the assay utilizes various antibodies which are bound to a solid support (e.g., one or more anti-core antibodies), and that detect antigen/antibody complexes formed when HCV infection is present in the sample. These antibodies may be polyclonal or monoclonal antibody preparations, monospecific antisera, human antibodies, or may be hybrid or chimeric antibodies, such as humanized antibodies, altered antibodies, $F(ab')_2$ fragments, F(ab) fragments, Fv fragments, single-domain antibodies, dimeric or trimeric antibody fragment constructs, minibodies, or functional fragments thereof which bind to the antigen in question.

Antibodies are produced using techniques well known to those of skill in the art and disclosed in, for example, U.S. Patent Nos. 4,011,308; 4,722,890; 4,016,043; 3,876,504; 3,770,380; and 4,372,745. For example, polyclonal antibodies are generated by immunizing a suitable animal, such as a mouse, rat, rabbit, sheep or goat, with an antigen of interest. In order to enhance immunogenicity, the antigen can be linked to a carrier prior to immunization. Such carriers are well known to those of ordinary skill in the art. Immunization is generally performed by mixing or emulsifying the antigen in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). The animal is generally boosted 2-6 weeks later with one or more injections of the antigen in saline, preferably using Freund's incomplete adjuvant. Antibodies may also be generated by *in vitro* immunization, using methods known in the art. Polyclonal antiserum is then obtained from the immunized animal. See, e.g., Houghton et al., U.S. Patent No. 5,350,671, for a description of the production of anti-HCV polyclonal

antibodies.

Monoclonal antibodies are generally prepared using the method of Kohler and Milstein (1975) *Nature* 256:495-497, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the antigen. B-cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (*e.g.*, hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (e.g., as ascites in mice).

The production of various anti-HCV monoclonal antibodies has been described in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., International Publication No. WO 93/00365; commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818; and Kashiwakuma et al., U.S. Patent No. 5,871,904.

As explained above, antibody fragments which retain the ability to recognize the antigen of interest, will also find use in the subject immunoassays. A number of antibody fragments are known in the art which comprise antigen-binding sites capable of exhibiting immunological binding properties of an intact antibody molecule. For example, functional antibody fragments can be produced by cleaving a constant region, not responsible for antigen binding, from the antibody molecule, using e.g., pepsin, to produce $F(ab')_2$ fragments. These fragments will contain two antigen binding sites, but lack a portion of the constant region from each of the heavy chains. Similarly, if

desired, Fab fragments, comprising a single antigen binding site, can be produced, e.g., by digestion of polyclonal or monoclonal antibodies with papain. Functional fragments, including only the variable regions of the heavy and light chains, can also be produced, using standard techniques such as recombinant production or preferential proteolytic cleavage of immunoglobulin molecules. These fragments are known as F_V. See, e.g., Inbar et al. (1972) *Proc. Nat. Acad. Sci. USA* 69:2659-2662; Hochman et al. (1976) *Biochem* 15:2706-2710; and Ehrlich et al. (1980) *Biochem* 19:4091-4096.

A single-chain Fv ("sFv" or "scFv") polypeptide is a covalently linked V_H-V_L heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85:5879-5883. A number of methods have been described to discern and develop chemical structures (linkers) for converting the naturally aggregated, but chemically separated, light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. The sFv molecules may be produced using methods described in the art. See, e.g., Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85:5879-5883; U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Design criteria include determining the appropriate length to span the distance between the C-terminus of one chain and the N-terminus of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Suitable linkers generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility.

"Mini-antibodies" or "minibodies" will also find use with the present invention. Minibodies are sFv polypeptide chains which include oligomerization domains at their C-termini, separated from the sFv by a hinge region. Pack et al. (1992) *Biochem* 31:1579-1584. The oligomerization domain comprises self-associating α-helices, e.g., leucine zippers, that can be further stabilized by additional disulfide bonds. The

oligomerization domain is designed to be compatible with vectorial folding across a membrane, a process thought to facilitate *in vivo* folding of the polypeptide into a functional binding protein. Generally, minibodies are produced using recombinant methods well known in the art. See, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126.

Production of Antigens for use in the HCV Immunoassays

As explained above, the molecules of the present invention are generally produced recombinantly. Thus, polynucleotides encoding HCV antigens for use with the present invention can be made using standard techniques of molecular biology. For example, polynucleotide sequences coding for the above-described molecules can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing the gene, or by deriving the gene from a vector known to include the same. Furthermore, the desired gene can be isolated directly from viral nucleic acid molecules, using techniques described in the art, such as in Houghton et al., U.S. Patent No. 5,350,671. The gene of interest can also be produced synthetically, rather than cloned. The molecules can be designed with appropriate codons for the particular sequence. The complete sequence is then assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; and Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Thus, particular nucleotide sequences can be obtained from vectors harboring the desired sequences or synthesized completely or in part using various oligonucleotide synthesis techniques known in the art, such as site-directed mutagenesis and polymerase chain reaction (PCR) techniques where appropriate. See, e.g., Sambrook, *supra*. In particular, one method of obtaining nucleotide sequences encoding the desired sequences is by annealing complementary sets of overlapping synthetic oligonucleotides produced in a conventional, automated polynucleotide synthesizer, followed by ligation with an appropriate DNA ligase and amplification of the ligated nucleotide sequence via PVR. See, e.g., Jayaraman et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:4084-4088.

Additionally, oligonucleotide directed synthesis (Jones et al. (1986) *Nature* 54:75-82), oligonucleotide directed mutagenesis of pre-existing nucleotide regions (Riechmann et al. (1988) *Nature* 332:323-327 and Verhoeyen et al. (1988) *Science* 239:1534-1536), and enzymatic filling-in of gapped oligonucleotides using T_4 DNA polymerase (Queen et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:10029-10033) can be used under the invention to provide molecules having altered or enhanced antigen-binding capabilities, and/or reduced immunogenicity.

Once coding sequences have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Suitable vectors include, but are not limited to, plasmids, phages, transposons, cosmids, chromosomes or viruses which are capable of replication when associated with the proper control elements.

The coding sequence is then placed under the control of suitable control elements, depending on the system to be used for expression. Thus, the coding sequence can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence of interest is transcribed into RNA by a suitable transformant. The coding sequence may or may not contain a signal peptide or leader sequence which can later be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector. For example, enhancer elements may be used herein to increase expression levels of the constructs. Examples include the SV40 early gene enhancer (Dijkema et al. (1985) *EMBO J.* 4:761), the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma

Virus (Gorman et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:6777) and elements derived from human CMV (Boshart et al. (1985) *Cell* 41:521), such as elements included in the CMV intron A sequence (U.S. Patent No. 5,688,688). The expression cassette may further include an origin of replication for autonomous replication in a suitable host cell, one or more selectable markers, one or more restriction sites, a potential for high copy number and a strong promoter.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the molecule of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences in the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

As explained above, it may also be desirable to produce mutants or analogs of the antigen of interest. This is particularly true with NS3/4a. Methods for doing so are described in, e.g., Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276. Mutants or analogs of this and other HCV proteins for use in the subject assays may be prepared by the deletion of a portion of the sequence encoding the polypeptide of interest, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, and the like, are well known to those skilled in the art. See, e.g., Sambrook et al., supra; Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. (1987) *BioTechniques* 5:786; Zoller and Smith (1983) *Methods Enzymol.* 100:468; Dalbie-McFarland et al. (1982) *Proc. Natl. Acad. Sci USA* 79:6409.

The molecules can be expressed in a wide variety of systems, including insect, mammalian, bacterial, viral and yeast expression systems, all well known in the art.

For example, insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). Similarly, bacterial and mammalian cell expression systems are well known in the art and described in, e.g., Sambrook et al., *supra*. Yeast expression systems are also known in the art and described in, e.g., *Yeast Genetic Engineering* (Barr et al., eds., 1989) Butterworths, London.

A number of appropriate host cells for use with the above systems are also known. For example, mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human embryonic kidney cells, human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Nucleic acid molecules comprising nucleotide sequences of interest can be stably integrated into a host cell genome or maintained on a stable episomal element in a suitable host cell using various gene delivery techniques well known in the art. *See, e.g.*, U.S. Patent No. 5,399,346.

Depending on the expression system and host selected, the molecules are

produced by growing host cells transformed by an expression vector described above under conditions whereby the protein is expressed. The expressed protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the product can be purified directly from the media. If it is not secreted, it can be isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The recombinant production of various HCV antigens has been described. See, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778.

Immunodiagnostic Assays

Once produced, the above anti-core antibodies and NS3/4a antigens are placed on an appropriate solid support for use in the subject immunoassays. A solid support, for the purposes of this invention, can be any material that is an insoluble matrix and can have a rigid or semi-rigid surface. Exemplary solid supports include, but are not limited to, substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. Particular supports include plates, pellets, disks, capillaries, hollow fibers, needles, pins, solid fibers, cellulose beads, pore-glass beads, silica gels, polystyrene beads optionally cross-linked with divinylbenzene, grafted co-poly beads, polyacrylamide beads, latex beads, dimethylacrylamide beads optionally crosslinked with N-N'-bis-acryloylethylenediamine, and glass particles coated with a hydrophobic polymer.

If desired, the molecules to be added to the solid support can readily be functionalized to create styrene or acrylate moieties, thus enabling the incorporation of the molecules into polystyrene, polyacrylate or other polymers such as polyimide, polyacrylamide, polyethylene, polyvinyl, polydiacetylene, polyphenylene-vinylene, polypeptide, polysaccharide, polysulfone, polypyrrole, polyimidazole, polythiophene,

polyether, epoxies, silica glass, silica gel, siloxane, polyphosphate, hydrogel, agarose, cellulose, and the like.

In one context, a solid support is first reacted with the HCV anti-core antibodies and NS3/4a epitope (collectively called "the solid-phase components" herein), and optionally, one or more MEFAs, under suitable binding conditions such that the molecules are sufficiently immobilized to the support. Sometimes, immobilization to the support can be enhanced by first coupling the antigen and/or antibody to a protein with better solid phase-binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other reagents that can be used to bind molecules to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. (1992) *Bioconjugate Chem.* 3:2-13; Hashida et al. (1984) *J. Appl. Biochem.* 6:56-63; and Anjaneyulu and Staros (1987) *International J. of Peptide and Protein Res.* 30:117-124.

After reacting the solid support with the solid-phase components, any nonimmobilized solid-phase components are removed from the support by washing, and the support-bound components are then contacted with a biological sample suspected of containing HCV antibodies and antigens (collectively called "ligand molecules" herein) under suitable binding conditions. After washing to remove any nonbound ligand molecules, a second anti-core antibody, directed against a different epitope than the anti-core antibody bound to the support, is added under suitable binding conditions. The added anti-core antibody includes a detectable label, as described above, and acts to bind any core antigen that might be present in the sample which has reacted with the support-bound anti-core antibody. Also added are one or more antigens that can react with antibodies present in the sample that have, in turn, reacted with the NS3/4A epitope. As explained above, the antigen is typically derived from the NS3 region of the HCV polyprotein, and particularly from the c33c region of HCV. See, Houghton et al., U.S.

Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci.* (1989) 89:10011-10015; International Publication No. WO 93/00365; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818, for a description of this region and epitopes derived therefrom. A labeled antibody directed against this antigen is also added. The antibody will therefore bind the antigen, which has reacted with anti-NS3 antibodies present in the sample. For this purpose, the c33c epitope can be conveniently provided as a fusion between c33c and human superoxide dismutase (hSOD), produced recombinantly e.g., by methods described in Houghton et al., U.S. Patent No. 5,350,671. The nucleotide and amino acid sequences for human SOD are known and reported in Hallewell et al., U.S. Patent No. 5,710,033. A labeled antibody directed against human SOD can therefore be used to detect the presence of complexes formed between the NS3/4a epitope, any antibodies in the sample which react with this epitope, and HCV polypeptides which in turn bind the antibody in the sample.

If a MEFA is present on the solid support, one or more additional antigens, reactive with antibodies from the biological sample which are bound to antigens present on the MEFA, may also be added to the assay. Particularly useful in this context is an antigen derived from the core region of HCV, and more particularly, from the c22 antigen which includes 119 N-terminal core amino acids of the HCV polyprotein. One particular antigen derived from c22 is c22ks Δ47-L44W which includes amino acids Lys_{10} to Ser_{99} of the polyprotein, as well as a deletion of Arg47 normally present and a substitution of Leu for Trp at position 44. As with the c33c epitope described above, this antigen can be provided as a fusion with hSOD and the same labeled antibody, directed against human SOD, can be used to detect the presence of complexes formed between antibodies present in the sample and the NS3/4a epitope and/or the MEFA, which complexes are also bound with the HCV antigens (e.g., c33c and c22).

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with the solid-phase components. A biological sample containing or suspected of containing ligand molecules is then added to the coated wells. After a period of incubation sufficient to allow ligand-molecule binding to the immobilized solid-phase component, the plate(s) can be washed to remove unbound

moieties and a detectably labeled secondary binding molecule (labeled anti-core antibody), an NS3 epitope-containing molecule, and an antibody directed against the NS3 epitope-containing molecule added. These molecules are allowed to react with any captured sample antigen and antibody, the plate washed and the presence of the labeled antibodies detected using methods well known in the art.

The above-described assay reagents, including the immunoassay solid support with bound antibodies and antigens, as well as antibodies and antigens to be reacted with the captured sample, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

HCV Antigen/Antibody Combination Immunoassay

The present HCV antigen/antibody combination immunoassay was compared to other HCV assays to test the seroconversion detection limits and compare these limits to those obtained in other commercially available assays as follows.

A. Materials and Methods

Blood Samples: Panels of commercially available human blood samples were used. Such panels are available from, e.g., Boston Biomedica, Inc., West Bridgewater, MA (BBI); Bioclinical Partners, Franklin, MA (BCP); and North American Biologics, Inc., BocoRatan, FL (NABI). The days indicated in Tables 5 and 6 are days on which blood was collected from the subjects.

Monoclonal Antibodies: Monoclonal antibodies c11-3, c11-7 and c11-14 were obtained from Ortho Clinical Diagnostics, Raritan, New Jersey. The c11-3 and c11-7 antibodies are directed against an N-terminal portion of the core (amino acids 10-53, numbered relative to the HCV1 polyprotein). Monoclonal antibody c11-14 is directed against a C-terminal portion of the core (amino acids 120-130, numbered relative to the HCV1 polyprotein). The c11-14 antibody was conjugated to horse radish peroxidase (HRP) using standard procedures.

Monoclonal antibody 5A-3 is an anti-SOD antibody directed against amino acids 1 to 65 of SOD and was made using standard techniques. The antibody was conjugated to HRP as described above.

B. Antigens:

The c33c antigen (266 amino acids, amino acids 1192 to 1457 of the HCV1 polyprotein) was expressed as an internal SOD fusion polypeptide in *E. coli* by methods described for the synthesis of the 5-1-1 antigen (Choo, et al., *Science* (1989) 244:359-362). The recombinant antigen was purified as described in Chien, et al., *Proc. Natl. Acad. Sci.* (1989) 89:10011-10015. See, also, Houghton et al., U.S. Patent No. 5,350,671, for production protocols for SOD-c33c.

The NS3/4a epitope used in the assay is a conformational epitope having the sequence specified in Figure 3.

C. Immunoassay Formats:

The Abbott PRISM assay (Abbott Laboratories, Abbott Park, IL), is commercially available and is an antibody-based detection assay. The assay was performed using the manufacturer's instructions.

The ORTHO HCV Version 3.0 ELISA Test System (termed Ortho 3.0 assay herein, Ortho Clinical Diagnostics, Raritan, New Jersey) is an antibody-based detection assay. The assay was conducted using the manufacturer's instructions.

The Roche Amplicor assay (Roche, Pleasant, CA) is a commercially available PCR-based assay. The assay was performed using the manufacturer's instructions.

The Gen-Probe TMA assay (San Diego, CA) is a commercially available transcription-mediated amplification assay. The assay was performed using the manufacturer's instructions.

The Ortho antigen assay (Ortho Clinical Diagnostics, Raritan, New Jersey) is an antigen-based detection assay. The assay was performed using the manufacturer's instructions.

The subject HCV antigen/antibody combination immunoassay was performed as follows. 4mg/mL each of purified monoclonal antibodies C11-7 and C11-3 in 1x phosphate-buffered saline (PBS), pH 7.4 were combined and mixed well. 90ng of the NS3/4a recombinant antigen was added to the same coating buffer. The solution was mixed for 30 minutes prior to coating. 200mL of the above solution was added per well to 96-well Costar medium binding microtiter plates (Corning, Inc.) Plates were incubated at 15-30°C for 16-24 hours. Plates were washed two times with dH_20, followed with 300µL/well postcoat buffer (1% bovine serum albumin (BSA), 1x PBS) for 1 hour and 300µl/well stability buffer (1x PBS, 1% BSA, mannitol, polyethylene glycol (PEG), gelatin) for 1 hour. Plates were aspirated and dried at 4°C in a lyophilizer for 24 hours. Plates were pouched with desiccant.

To conduct the antigen/antibody combination immunoassay, 100µL of enhanced lysis buffer (1% N-laurylsarcosine, 0.65M NaCl, 50mg/mL mouse IgG technical grade (Sigma, St. Louis, MO), 1% BSA sulfhydryl-modified (Bayer), 0.1% Casein) were added to the plate. 100mL of sample were then added. This was incubated on a shaker at 40°C for one hour. The plates were washed six times with 1x PBS, 0.1% Tween-20,

on an Ortho Plate Washer. 200mL conjugate solution (1:75 dilution c11-14-HRP with 250ng/assay SOD-c33c antigen plus 1:5000 dilution mouse anti-SOD-HRP in HCV 3.0 sample diluent (from ORTHO HCV Version 3.0 ELISA Test System, Ortho Clinical Diagnostics, Raritan, New Jersey) without SOD extract, all prepared 30 minutes prior to addition). The solution was incubated 45 minutes with shaking at 40°C. This was washed six times, as above, and 200mL substrate solution (1 OPD tablet/10mL) was added. The OPD tablet contains *o*-phenylenediamine dihydrochloride and hydrogen peroxide for horse radish peroxidase reaction color development and is available from Sigma, St. Louis, MO. This was incubated 30 minutes at 15-30°C in the dark. The reaction was stopped by addition of 50mL 4N H_2SO_4 and the plates were read at 492nm, relative to absorbance at 690nm as control.

D. Results:

The results of the various assays are shown in Tables 5 and 6, which depict two separate experiments done on blood samples exposed to HCV infection as indicated. Shaded areas indicate detection of virus. As shown in below, Chiron's combination antigen/antibody assay detected seroconversion in all samples, while all other antibody- and antigen-based assays failed to detect seroconversion in at least one sample. In particular, neither of the antibody-based assays detected seroconversion until at least day 18 (Table 5). Table 6 shows that neither of the antibody-based assays detected the presence of HCV infection at day 22. Moreover, the Ortho antigen-based assay failed to detect seroconversion from days 85 on.

Thus, based on the above results, it is clear that the novel combination antibody/antigen assay reduces the number of false negatives obtained using other conventional antibody- and antigen-based assays.

Table 5
HCV Seroconversion

Days	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	$>5 \times 10^5$	9.25	18.6	2.8
4	0.1	0.0	$>5 \times 10^5$	9.29	19.0	3.1
7	0.1	0.0	$>5 \times 10^5$	9.52	22.3	1.5
13	0.3	0.1	$>5 \times 10^5$	9.59	26.2	1.7
18	1.3	0.4	$>5 \times 10^5$	9.70	15.9	1.2
21	2.2	1.0	$>5 \times 10^5$	9.39	11.3	1.5
164	4.2	4.4	4×10^6	9.28	0.11	2.5

Table 6
HCV Seroconversion

Days	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	BLD		0.11	0.5
13	0.1	0.0	$>5 \times 10^5$		44.0	3.0
20	0.1	0.0	$>5 \times 10^5$		24.2	1.3
22	0.3	0.0	$>5 \times 10^5$		29.2	1.6
85	5.4	4.7	BQR		0.06	1.1
131	4.3	4.7	BQR		0.09	1.0
135	4.6	4.7	3×10^3		0.09	1.2
138	5.5	4.7	BLD		0.08	1.2
146	5.9	4.7	BLD		0.11	2.1
152	5.2	4.7	BQR		0.07	1.8

EXAMPLE 2

Production of an NS3/4a Conformational Epitope with

Thr to Pro and Ser to Ile Substitutions

A conformational epitope of NS3/4a was obtained as follows. This epitope has the sequence specified in Figures 4A through 4D and differs from the native sequence at

would provide a 5' *Hind*III cloning site, followed by the sequence ACAAAACAAA, the initiator ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*I site at amino acid 1046; (b) a 683 bp *Bgl*I-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI; and (c) a pSP72 vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65332) which had been digested with *Hind*III and *Cla*I, dephosphorylated, and gel-purified. Plasmid pAcHLTns3ns4aPI was derived from pAcHLT, a baculovirus expression vector commercially available from BD Pharmingen (San Diego, CA). In particular, a pAcHLT *Eco*RI-*Pst*I vector was prepared, as well as the following fragments: *Eco*RI- *Alwn*I, 935 bp, corresponding to amino acids 1027-1336 of the HCV-1 genome; *Alwn*I-*Sac*II, 247 bp, corresponding to amino acids 1336-1419 of the HCV-1 genome; *Hinf*I-*Bgl*I, 175 bp, corresponding to amino acids 1449-1509 of the HCV-1 genome; *Bgl*I-*Pst*I, 619 bp, corresponding to amino acids 1510-1711 of the HCV-1 genome, plus the transcription termination codon. A *Sac*II -*Hinf*I synthetically generated fragment of 91 bp, corresponding to amino acids 1420-1448 of the HCV-1 genome and containing the PI mutations (Thr-1428 mutated to Pro, Ser-1429 mutated to Ile), was ligated with the 175 bp *Hinf*I-*Bgl*I fragment and the 619 bp *Bgl*I-*Pst*I fragment described above and subcloned into a pGEM-5Zf(+) vector digested with *Sac*II and *Pst*I. pGEM-5Zf(+) is a commercially available *E. coli* vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65308). After transformation of competent HB101 cells, miniscreen analysis of individual clones and sequence verification, an 885 bp *Sac*II-*Pst*I fragment from pGEM5.PI clone2 was gel-purified. This fragment was ligated with the *Eco*RI-*Alwn*I 935 bp fragment, the *Alwn*I-*Sac*II 247 bp fragment and the pAcHLT *Eco*RI-*Pst*I vector, described above. The resultant construct was named pAcHLTns3ns4aPI.

The ligation mixture above was transformed into HB101-competent cells and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual clones led to the identification of putative positives, two of which were amplified. The plasmid DNA for pSP72 1aHC, clones #1 and #2 were prepared with a Qiagen Maxiprep kit and were sequenced.

Next, the following fragments were ligated together: (a) a 761 bp *Hind*III-*Cla*I

fragment from pSP721aHC #1 (pSP72.1aHC was generated by ligating together the following: pSP72 which had been digested with *Hind*III and *Cla*I, synthetic oligonucleotides which would provide a 5' *Hind*III cloning site, followed by the sequence ACAAAACAAA, the initiation codon ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*II site at amino acid 1046, and a 683 bp *Bgl*II-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI); (b) a 1353 bp *Bam*HI-*Hind*III fragment for the yeast hybrid promoter ADH2/GAPDH; (c) a 1320 bp *Cla*I-*Sal*I fragment (encoding HCV1a amino acids 1046-1711 with Thr 1428 mutated to Pro and Ser 1429 mutated to Ile) from pAcHLTns3ns4aPI; and (d) the pBS24.1 yeast expression vector which had been digested with *Bam*HI and *Sal*I, dephosphorylated and gel-purified. The ligation mixture was transformed into competent HB101 and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual colonies led to the identification of clones with the expected 3446 bp *Bam*HI-*Sal*I insert which was comprised of the ADH2/GAPDH promoter, the initiator codon ATG and HCV1a NS3/4a from amino acids 1027-1711 (shown as amino acids 1-686 of Figures 4A-4D), with Thr 1428 (amino acid position 403 of Figures 4A-4D) mutated to Pro and Ser 1429 (amino acid position 404 of Figures 4A-4D) mutated to Ile. The construct was named pd.HCV1a.ns3ns4aPI (see, Figure 5).

S. cerevisiae strain AD3 was transformed with pd.HCV1a.ns3ns4aPI and single transformants were checked for expression after depletion of glucose in the medium. The recombinant protein was expressed at high levels in yeast, as detected by Coomassie blue staining and confirmed by immunoblot analysis using a polyclonal antibody to the helicase domain of NS3.

EXAMPLE 3

Purification of NS3/4a Conformational Epitope

The NS3/4a conformational epitope was purified as follows. *S. cerevisiae* cells from above, expressing the NS3/4a epitope were harvested as described above. The cells were suspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin) and lysed in a Dyno-Mill (Wab Willy A. Bachofon, Basel, Switzerland) or equivalent apparatus using glass beads, at a ratio of 1:1:1 cells:buffer:0.5 mm glass beads. The lysate was centrifuged at 30100 x g for 30 min at 4°C and the pellet containing the insoluble protein fraction was added to wash buffer (6 ml/g start cell pellet weight) and rocked at room temperature for 15 min. The wash buffer consisted of 50 mM $NaPO_4$ pH 8.0, 0. 3 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 0.05% octyl glucoside, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. Cell debris was removed by centrifugation at 30100 x g for 30 min at 4°C. The supernatant was discarded and the pellet retained.

Protein was extracted from the pellet as follows. 6 ml/g extraction buffer was added and rocked at room temperature for 15 min. The extraction buffer consisted of 50 mM Tris pH 8.0, 1 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. This was centrifuged at 30100 x g for 30 min at 4°C. The supernatant was retained and ammonium sulfate added to 17.5% using the following formula: volume of supernatant (ml) multiplied by x% ammonium sulfate/(1 - x% ammonium sulfate) = ml of 4.1 M saturated ammonium sulfate to add to the supernatant. The ammonium sulfate was added dropwise while stirring on ice and the solution stirred on ice for 10 min. The solution was centrifuged at 17700 x g for 30 min at 4°C and the pellet retained and stored at 2°C to 8°C for up to 48 hrs.

The pellet was resuspended and run on a Poly U column (Poly U Sepharose 4B, Amersham Pharmacia) at 4°C as follows. Pellet was resuspended in 6 ml Poly U equilibration buffer per gram of pellet weight. The equilibration buffer consisted of 25 mM HEPES pH 8.0, 200 mM NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. The solution was rocked at 4°C for 15 min and centrifuged at 31000 x g for 30 min at 4°C.

A Poly U column (1 ml resin per gram start pellet weight) was prepared. Linear flow rate was 60 cm/hr and packing flow rate was 133% of 60 cm/hr. The column was equilibrated with equilibration buffer and the supernatant of the resuspended ammonium sulfate pellet was loaded onto the equilibrated column. The column was washed to baseline with the equilibration buffer and protein eluted with a step elution in the following Poly U elution buffer: 25 mM HEPES pH 8.0, 1 M NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. Column eluate was run on SDS-PAGE (Coomassie stained) and aliquots frozen and stored at -80°C. The presence of the NS3/4a epitope was confirmed by Western blot, using a polyclonal antibody directed against the NS3 protease domain and a monoclonal antibody against the 5-1-1 epitope (HCV 4a).

Additionally, protease enzyme activity was monitored during purification as follows. An NS4A peptide (KKGSVVIVGRIVLSGKPAIIPKK), and the sample containing the NS3/4a conformational epitope, were diluted in 90 µl of reaction buffer (25 mM Tris, pH 7.5, 0.15M NaCl, 0.5 mM EDTA, 10% glycerol, 0.05 n-Dodecyl B-D-Maltoside, 5 mM DTT) and allowed to mix for 30 minutes at room temperature. 90 µl of the mixture were added to a microtiter plate (Costar, Inc., Corning, NY) and 10 µl of HCV substrate (AnaSpec, Inc., San Jose CA) was added. The plate was mixed and read on a Fluostar plate reader. Results were expressed as relative fluorescence units (RFU) per minute.

Using these methods, the product of the 1 M NaCl extraction contained 3.7 RFU/min activity, the ammonium sulfate precipitate had an activity of 7.5 RFU/min and the product of the Poly U purification had an activity of 18.5 RFU/min.

EXAMPLE 4

Competition Studies

The following competition study was conducted in order to assess whether the NS3/4a conformational epitope detected different antibodies than other HCV antigens. In particular, the NS3/4a antigen was compared with the c200 antigen as follows.

0.5 µg and 1.0 µg of NS3/4a, produced as described above, or c200 (*Hepatology*

(1992) 15:19-25, available in the ORTHO HCV Version 3.0 ELISA Test System, Ortho-Clinical Diagnostics, Raritan, New Jersey), were mixed with 20 µl of sample PHV914-5 (an early seroconversion bleed obtained from blood of an infected individual) in a total volume of 220 µl (1 x PBS). The mixture was incubated for 1 hour in microwells at 37°C. The mixture was then transferred to NS3/4a-coated plates and incubated for 1 hour at 37°C. Plates were washed and assayed as follows.

1 µg of c200 antigen was added to 10 µl of sample PHV914-5 in a total volume of about 220 µl. The mixture was incubated for 1 hour in a micro well at 37°C and 200 µl transferred to an NS3/4a-coated plate (100 ng/assay) and incubated for 1 hour at 37°C. Plates were washed five times with 1 x PBS, 0.1% Tween-20. 200 µl of conjugate solution (described above) were added, and the plates incubated and assayed. Controls which consisted of PHV914-5 and 1 x PBS (without antigen) were also treated as above.

Results are shown in Table 7. Percent inhibition results shown in column 4 are calculated as column 3 minus (column 2 divided by column 3 times 100). As can be seen, the data show that NS34a is neutralized by early seroconversion antibodies and c200 is not. A strong signal was achieved when antibodies in PHV914-5 c33c early seroconversion panel member reacted with the NS34a coated on the plate. The c200 antigen was not neutralized by these antibodies. This is shown in the top panel of Table 7. When NS34a was mixed with the PHV914-5 sample, it was neutralized and therefore no antibodies were present in the sample to react with NS34a that was coated on the microplate. The data indicate that NS34a may be detecting a different class of antibodies than is detected by c200.

Competition Studies to Show NS34a Antigen Detects Different Antibodies in Early c33c Seroconversion Panel Compared to c200 Antigen

		1	2	3	4
				Control 1XPBS	% Inhibition
c200	+	PHV914-5			
		s		s	
1ug		1.450		1.645	12
1ug		1.545		1.687	8
0.5ug		1.557		1.913	19
0.5ug		1.719		1.804	5
NS3/4a	+	PHV914-5			
		s		s	
1ug		0.054		1.599	97
1ug		0.037		1.677	98
0.5ug		0.066		1.672	96
0.5ug		NA		1.524	NA

TABLE 7

EXAMPLE 5

Stability Studies of NS3/4a Conformational Epitope

To assess the role of stability of the NS3/4a epitope to assay performance, the following study was done to determine NS3/4a immunoreactivity versus time at room temperature. Small aliquots of stock NS3/4a were allowed to sit at room temperature and then frozen at intervals as shown in Table 8. All vials were coated simultaneously and tested against two early NS3 seroconversion panels.

As can be seen in Table 8, the NS3/4a stock is not stable and immunoreactivity decreases with time. In addition, maintaining NS3/4a conformation is necessary for immunoreactivity.

Further stability studies were conducted as follows. Two conformational monoclonal antibodies made against NS3/4a using standard procedures were substituted for anti-HCV early seroconversion panels. Stock NS3/4a vials were stored at room temperature at time intervals 3, 6 and 24 hours. The NS3/4a from the frozen vials was coated at 90 ng/ml and assayed using the procedure described above. Results suggested that the two monoclonals were indeed conformational and their reactivity was sensitive to the handling of stock NS3/4a antigen at room temperature. The reactivity of a positive control monoclonal antibody did not change.

Time (hrs)	0		6		21.4		29		35.5		46		52		control	
	A		D		G		H		I		K		N		Reference	
	s/co		s/co		s/co		s/co		s/co		s/co		s/co		s/co	
PHV 904-1	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 904-2	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 904-3	7.5		0.3		0.1		0.0		0.0		0.0		0.0		1.8	
PHV 904-4	3.7		1.0		0.2		0.1		0.1		0.1		0.1		1.4	
PHV 904-5	1.4		2.0		0.7		0.6		0.3		0.2		0.3		1.5	
PHV 904-6	5.7		2.8		1.1		1.0		0.6		0.5		0.6		1.8	
PHV 904-7	5.1		3.4		1.5		1.0		1.1		0.5		0.7		5.1	
PHV 914-1	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 914-2	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	
PHV 914-3	0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.7	
PHV 914-4	0.5		0.1		0.0		0.0		0.0		0.0		0.0		1.3	
PHV 914-5	2.1		0.4		0.0		0.0		0.0		0.0		0.0		3.1	
PHV 914-6	2.3		0.4		0.0		0.0		0.0		0.0		0.0		2.9	
PHV 914-7	2.3		0.5		0.1		0.1		0.0		0.0		0.0		2.5	
PHV 914-8	2.9		0.7		0.1		0.1		0.1		0.1		0.1		1.6	
Enzyme RFU/min	8.75		4.14		3.08		1.88		1.75		1.75		0.75			

TABLE 8

EXAMPLE 6

Immunoreactivity of NS3/4a Conformational Epitope Verus Denatured NS3/4a

The immunoreactivity of the NS3/4a conformational epitope, produced as described above, was compared to NS3/4a which had been denatured by adding SDS to the NS3/4a conformational epitope preparation to a final concentration of 2%. The denatured NS3/4a and conformational NS3/4a were coated onto microtiter plates as described above. The c200 antigen (*Hepatology* (1992) 15:19-25, available in the ORTHO HCV Version 3.0 ELISA Test System, Ortho-Clinical Diagnostics, Raritan, New Jersey) was also coated onto microtiter plates. The c200 antigen was used as a comparison it is presumed to be non-conformational due to the presence of reducing agent (DTT) and detergent (SDS) in its formulation.

The immunoreactivity was tested against two early HCV seroconversion panels, PHV 904 and PHV 914 (commercially available human blood samples from Boston Biomedica, Inc., West Bridgewater, MA). The results are shown in Table 9. The data suggest that the denatured or linearized form of NS3/4a (as well as c200) does not detect early seroconversion panels as early as the NS3/4a conformational epitope.

	NS3/4a vs. denatured NS3/4a						
	*Spiked 2% SDS to stock NS3/4a						
		NS3/4a	dNS3/4a*	c200	NS3/4a	dNS3/4a*	c200
		OD	OD	OD	s/co	s/co	s/co
HCV	PHV 904-1	0.012	0.012	0.009	0.02	0.02	0.01
Seroconversions	PHV 904-2	0.011	0.009	0.008	0.02	0.01	0.01
	PHV 904-3	1.124	0.071	0.045	1.80	0.11	0.07
	PHV 904-4	2.401	0.273	0.129	3.85	0.44	0.21
	PHV 904-5	3.022	0.793	0.347	4.85	1.28	0.57
	PHV 904-6	2.711	1.472	0.774	4.35	2.37	1.26
	PHV 904-7	3.294	1.860	0.943	5.29	3.00	1.55
	PHV 914-1	0.006	0.004	0.001	0.01	0.01	0.00
	PHV 914-2	0.005	0.004	0.002	0.01	0.01	0.00
	PHV 914-3	0.098	0.003	0.001	0.16	0.00	0.00
	PHV 914-4	1.118	0.006	0.004	1.79	0.01	0.01
	PHV 914-5	2.035	0.044	0.022	3.26	0.07	0.04
	PHV 914-6	2.092	0.074	0.025	3.35	0.12	0.04
	PHV 914-7	2.519	0.281	0.132	4.03	0.45	0.22
	PHV 914-8	2.746	0.907	0.500	4.40	1.46	0.82
	PHV 914-9	3.084	1.730	0.931	4.94	2.78	1.53
HCV 3.0	Neg.Cont.	0.023	0.024	0.008			
Controls	Neg.Cont.	0.027	0.024	0.007			
	Neg.Cont.	0.021	0.017	0.005			
	average	0.024	0.022	0.007			
	cutoff	0.624	0.622	0.607			
	Pos. Cont.	1.239	0.903	0.575	1.99	1.45	0.95
	Pos. Cont.	1.445	0.916	0.614	2.32	1.47	1.01

TABLE 9

Immunoreactivity of the conformational epitope was also tested using monoclonal antibodies to NS3/4a, made using standard procedures. These monoclonal antibodies were then tested in the ELISA format against NS3/4a and denatured NS3/4a and c200 antigen. The data show that anti-NS3/4a monoclonals react to the NS3/4a and denatured NS3/4a in a similar manner to the seroconversion panels shown in Table 10. This result also provides further evidence that the NS3/4a is conformational in nature as monoclonal antibodies can be made which are similar in reactivity to the early c33c seroconversion panels .

Table 10

		Plate		
		NS3/4a	dNS3/4a	c200
Monoclonal		OD	OD	OD
4B9/E3	1:100	1.820	0.616	0.369
	1:1000	1.397	0.380	0.246
	1:10000	0.864	0.173	0.070
	1:20000	0.607	0.116	0.085
5B7/D7	1:100	2.885	0.898	0.436
	1:1000	2.866	0.541	0.267
	1:10000	1.672	0.215	0.086
	1:20000	1.053	0.124	0.059
1A8/H2	1:100	1.020	0.169	0.080
	1:1000	0.921	0.101	0.043
	1:10000	0.653	0.037	0.013
	1:20000	0.337	0.027	0.011

Accordingly, novel HCV detection assays have been disclosed. From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the disclosure herein.

5

Claims

1. An immunoassay solid support comprising at least one hepatitis C virus (HCV) anti-core antibody and at least one isolated HCV NS3/4a epitope bound thereto.

2. The immunoassay solid support of claim 1, comprising at least two HCV anti-core antibodies bound thereto.

3. The immunoassay solid support of claim 1, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen.

4. The immunoassay solid support of claim 3, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence.

5. The immunoassay solid support of claim 1, wherein said at least one anti-core antibody is a monoclonal antibody.

6. The immunoassay solid support of claim 1, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

7. The immunoassay solid support of claim 1, further comprising a multiple epitope fusion antigen bound thereto.

8. The immunoassay solid support of claim 7, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F.

9. An immunoassay solid support comprising two hepatitis C virus (HCV) anti-core monoclonal antibodies and an HCV NS3/4a conformational epitope comprising the

amino acid sequence depicted in Figures 4A-4D, bound thereto.

10. The immunoassay solid support of claim 9, wherein said two anti-core antibodies are directed against an N-terminal region of the HCV core antigen.

11. The immunoassay solid support of claim 10, wherein said two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence.

12. An immunoassay solid support comprising two hepatitis C virus (HCV) anti-core monoclonal antibodies, an HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, and a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, bound thereto.

13. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 1;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to said at least one anti-core antibody and said NS3/4a epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) an antigen that reacts with an HCV antibody from the biological sample reactive with said NS3/4a epitope; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with the antigen of (ii);

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

14. The method of claim 13, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

15. The method of claim 14, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

16. The method of claim 13, wherein said antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c33c region of the HCV polyprotein.

17. The method of claim 16, wherein the c33c epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

18. The method of claim 13, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

19. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 2;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies and said NS3/4a epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is

directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequence;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

20. The method of claim 19, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

21. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 9;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies and said NS3/4a conformational epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequence;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

22. The method of claim 21, wherein said at least two anti-core antibodies are

directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

23. The method of claim 22, wherein said at least two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

24. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 7;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to said at least one anti-core antibody, said NS3/4a epitope, and said multiple epitope fusion antigen;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) first and second antigens that react with an HCV antibody from the biological sample reactive with said NS3/4a epitope and said multiple epitope fusion antigen, respectively; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with the antigens of (ii);

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

25. The method of claim 24, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen and said first detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core

antigen.

26. The method of claim 25, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

27. The method of claim 24, wherein said first antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c33c region of the HCV polyprotein.

28. The method of claim 27, wherein the c33c epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

29. The method of claim 24, wherein said second antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c22 region of the HCV polyprotein.

30. The method of claim 29, wherein the epitope from the c22 region comprises amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence, wherein said epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

31. The method of claim 24, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F.

32. A method of detecting hepatitis C virus (HCV) infection in a biological

sample, said method comprising:

(a) providing an immunoassay solid support according to claim 12;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies, said NS3/4a conformational epitope, and said multiple epitope fusion antigen, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence and an epitope from the c22 region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequences;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

33. The method of claim 32, wherein said at least two anti-core antibodies are directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

34. The method of claim 33, wherein said at least two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

35. The method of claim 32, wherein the epitope from the c22 region comprises amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a

substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence.

36. An immunodiagnostic test kit comprising the immunoassay solid support of any of claims 1-12, and instructions for conducting the immunodiagnostic test.

37. A method of producing an immunoassay solid support, comprising:
(a) providing a solid support; and
(b) binding at least one hepatitis C virus (HCV) anti-core antibody and at least one isolated HCV NS3/4a conformational epitope thereto.

38. A method of producing an immunoassay solid support, comprising:
(a) providing a solid support; and
(b) binding two hepatitis C virus (HCV) anti-core antibodies and an isolated HCV NS3/4a conformational epitope thereto.

39. The method of either of claims 38 or 39, further comprising binding at least one multiple epitope fusion antigen to the solid support.

40. A multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 80% sequence identity thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

41. The multiple epitope fusion antigen of claim 40, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 90% sequence identity thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

42. The multiple epitope fusion antigen of claim 40, wherein said multiple epitope fusion antigen consists of the amino acid sequence depicted in Figures 5A-5F.

43. A polynucleotide comprising a coding sequence for the multiple epitope fusion antigen of any of claims 40-42.

44. A recombinant vector comprising:

(a) a polynucleotide according to claim 43;

(b) and control elements operably linked to said polynucleotide whereby the coding sequence can be transcribed and translated in a host cell.

45. A host cell transformed with the recombinant vector of claim 44.

46. A method of producing a recombinant multiple epitope fusion antigen comprising:

(a) providing a population of host cells according to claim 45; and

(b) culturing said population of cells under conditions whereby the multiple epitope fusion antigen encoded by the coding sequence present in said recombinant vector is expressed.

HCV Genome and Recombinant Proteins

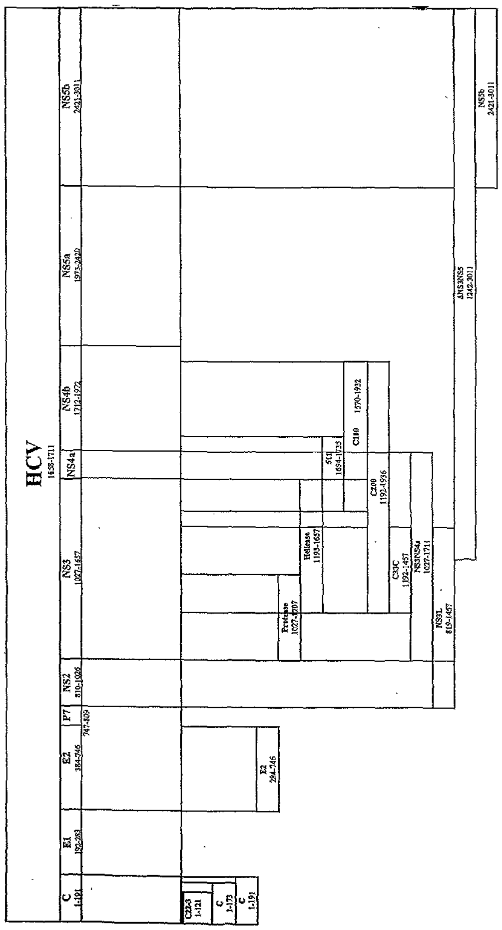


FIG. 1

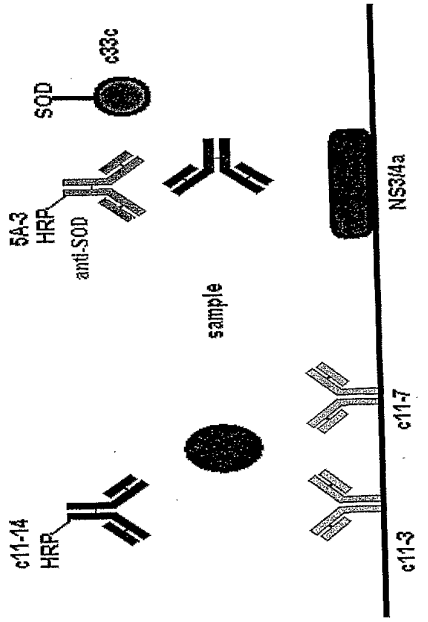


FIG. 2

```
MSPIDPMGHHHHHHGRRRASVAAGILVPRGSPGLDGICSIEEFAPITAYAQQTRGLLGCIITSLTGRDKNQVE  73
GEVQIVSTAAQTFLATCINGVCWTVYHGAGTRTIASPKGPVIQMYTNVDQDLVGWPASQGTRSLTPCTCGSSD 146
LYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSAGGPLLCPAGHAVGIFRAAVCTRGVAKAVDFIPVENLE 219
TTMRSPVFTDNSSPPVVPQSFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHGID 292
PNIRTGVRTITTGSPITYSTYGKFLADGGCSGGAYDIIICDECHSTDATSILGIGTVLDQAETAGARLVVLAT 365
ATPPGSVTVPHPNIEEVALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKLVALGINAVAYYRGL 438
DVSVIPPIGDVVVVATDALMTGYTGDFDSVIDCNTCVTQTVDFSLDPTFTIETITLPQDAVSRTQRRGRTGRG 511
KPGIYRFVAPGERPSGMFDSSVLCECYDAGCAWYELTPAETTVRLRAYMNTPGLPVCQDHLEFWEGVFTGLTH 584
IDAHFLSQTKQSGENLPYLVAYQATVCARAQAPPPSWDQMWKCLIRLKPTLHGPTPLLYRLGAVQNEITLTHP 657
VTKYIMTCMSADLEVVTSTWVLVGGVLAALAAYCLSTGCVVIVGRVVLSGKPAIIPDREVLYREFDEMEEC  728
```

FIG. 3

```
                              1                                   10
                              M   A   P   I   T   A   Y   A   Q   Q
                              ATG GCG CCC ATC ACG GCG TAC GCC CAG CAG

                                                20
T   R   G   L   L   G   C   I   I   T   S   L   T   G   R
ACA AGG GGC CTC CTA GGG TGC ATA ATC ACC AGC CTA ACT GGC CGG

                30                                          40
D   K   N   Q   V   E   G   E   V   Q   I   V   S   T   A
GAC AAA AAC CAA GTG GAG GGT GAG GTC CAG ATT GTG TCA ACT GCT

                                    50
A   Q   T   F   L   A   T   C   I   N   G   V   C   W   T
GCC CAA ACC TTC CTG GCA ACG TGC ATC AAT GGG GTG TGC TGG ACT

                60                                          70
V   Y   H   G   A   G   T   R   T   I   A   S   P   K   G
GTC TAC CAC GGG GCC GGA ACG AGG ACC ATC GCG TCA CCC AAG GGT

                                    80
P   V   I   Q   M   Y   T   N   V   D   Q   D   L   V   G
CCT GTC ATC CAG ATG TAT ACC AAT GTA GAC CAA GAC CTT GTG GGC

                90                                          100
W   P   A   P   Q   G   S   R   S   L   T   P   C   T   C
TGG CCC GCT CCG CAA GGT AGC CGA TCA TTG ACA CCC TGC ACT TGC

                                    110
G   S   S   D   L   Y   L   V   T   R   H   A   D   V   I
GGC TCC TCG GAC CTT TAC CTG GTC ACG AGG CAC GCC GAT GTC ATT

                120                                         130
P   V   R   R   R   G   D   S   R   G   S   L   L   S   P
CCC GTG CGC CGG CGG GGT GAT AGC AGG GGC AGC CTG CTG TCG CCC

                                    140
R   P   I   S   Y   L   K   G   S   S   G   G   P   L   L
CGG CCC ATT TCC TAC TTG AAA GGC TCC TCG GGG GGT CCG CTG TTG

                150                                         160
C   P   A   G   H   A   V   G   I   F   R   A   A   V   C
TGC CCC GCG GGG CAC GCC GTG GGC ATA TTT AGG GCC GCG GTG TGC

                                    170
T   R   G   V   A   K   A   V   D   F   I   P   V   E   N
ACC CGT GGA GTG GCT AAG GCG GTG GAC TTT ATC CCT GTG GAG AAC

                180                                         190
L   E   T   T   M   R   S   P   V   F   T   D   N   S   S
CTA GAG ACA ACC ATG AGG TCC CCG GTG TTC ACG GAT AAC TCC TCT
```

FIG. 4A

```
                                                200
 P   P   V   V   P   Q   S   F   Q   V   A   H   L   H   A
CCA CCA GTA GTG CCC CAG AGC TTC CAG GTG GCT CAC CTC CAT GCT

                    210                                 220
 P   T   G   S   G   K   S   T   K   V   P   A   A   Y   A
CCC ACA GGC AGC GGC AAA AGC ACC AAG GTC CCG GCT GCA TAT GCA

                                                230
 A   Q   G   Y   K   V   L   V   L   N   P   S   V   A   A
GCT CAG GGC TAT AAG GTG CTA GTA CTC AAC CCC TCT GTT GCT GCA

                    240                                 250
 T   L   G   F   G   A   Y   M   S   K   A   H   G   I   D
ACA CTG GGC TTT GGT GCT TAC ATG TCC AAG GCT CAT GGG ATC GAT

                                                260
 P   N   I   R   T   G   V   R   T   I   T   T   G   S   P
CCT AAC ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT GGC AGC CCC

                    270                                 280
 I   T   Y   S   T   Y   G   K   F   L   A   D   G   G   C
ATC ACG TAC TCC ACC TAC GGC AAG TTC CTT GCC GAC GGC GGG TGC

                                                290
 S   G   G   A   Y   D   I   I   I   C   D   E   C   H   S
TCG GGG GGC GCT TAT GAC ATA ATA ATT TGT GAC GAG TGC CAC TCC

                    300                                 310
 T   D   A   T   S   I   L   G   I   G   T   V   L   D   Q
ACG GAT GCC ACA TCC ATC TTG GGC ATT GGC ACT GTC CTT GAC CAA

                                                320
 A   E   T   A   G   A   R   L   V   V   L   A   T   A   T
GCA GAG ACT GCG GGG GCG AGA CTG GTT GTG CTC GCC ACC GCC ACC

                    330                                 340
 P   P   G   S   V   T   V   P   H   P   N   I   E   E   V
CCT CCG GGC TCC GTC ACT GTG CCC CAT CCC AAC ATC GAG GAG GTT

                                                350
 A   L   S   T   T   G   E   I   P   F   Y   G   K   A   I
GCT CTG TCC ACC ACC GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC

                    360                                 370
 P   L   E   V   I   K   G   G   R   H   L   I   F   C   H
CCC CTC GAA GTA ATC AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT

                                                380
 S   K   K   K   C   D   E   L   A   A   K   L   V   A   L
TCA AAG AAG AAG TGC GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG
```

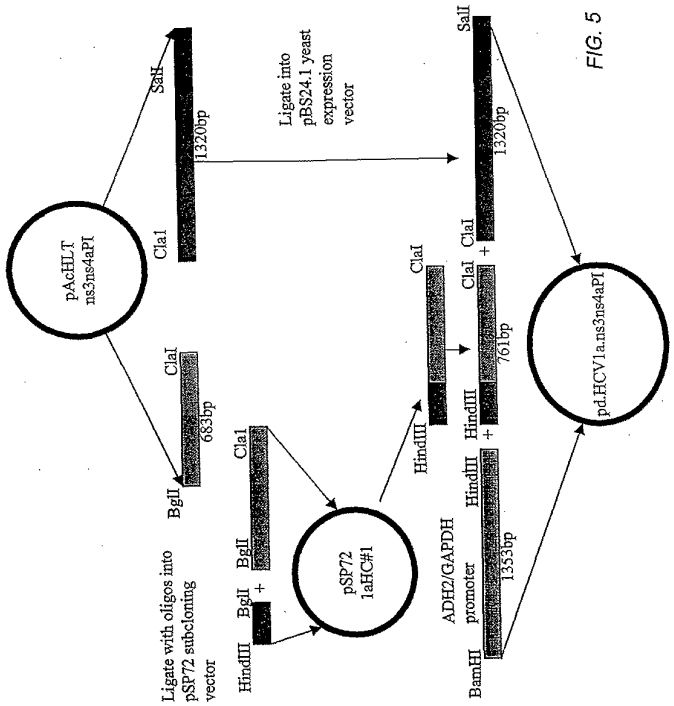
FIG. 4B

```
                390                                                 400
 G  I  N  A  V  A  Y  Y  R  G  L  D  V  S  V
GGC ATC AAT GCC GTG GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC

                                   410
 I  P  P  I  G  D  V  V  V  V  A  T  D  A  L
ATC CCG CCC ATC GGC GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC

                420                                                 430
 M  T  G  Y  T  G  D  F  D  S  V  I  D  C  N
ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT

                                   440
 T  C  V  T  Q  T  V  D  F  S  L  D  P  T  F
ACG TGT GTC ACC CAG ACA GTC GAT TTC AGC CTT GAC CCT ACC TTC

                450                                                 460
 T  I  E  T  I  T  L  P  Q  D  A  V  S  R  T
ACC ATT GAG ACA ATC ACG CTC CCC CAA GAT GCT GTC TCC CGC ACT

                                   470
 Q  R  R  G  R  T  G  R  G  K  P  G  I  Y  R
CAA CGT CGG GGC AGG ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA

                480                                                 490
 F  V  A  P  G  E  R  P  S  G  M  F  D  S  S
TTT GTG GCA CCG GGG GAG CGC CCC TCC GGC ATG TTC GAC TCG TCC

                                   500
 V  L  C  E  C  Y  D  A  G  C  A  W  Y  E  L
GTC CTC TGT GAG TGC TAT GAC GCA GGC TGT GCT TGG TAT GAG CTC

                510                                                 520
 T  P  A  E  T  T  V  R  L  R  A  Y  M  N  T
ACG CCC GCC GAG ACT ACA GTT AGG CTA CGA GCG TAC ATG AAC ACC

                                   530
 P  G  L  P  V  C  Q  D  H  L  E  F  W  E  G
CCG GGG CTT CCC GTG TGC CAG GAC CAT CTT GAA TTT TGG GAG GGC

                540                                                 550
 V  F  T  G  L  T  H  I  D  A  H  F  L  S  Q
GTC TTT ACA GGC CTC ACT CAT ATA GAT GCC CAC TTT CTA TCC CAG

                                   560
 T  K  Q  S  G  E  N  L  P  Y  L  V  A  Y  Q
ACA AAG CAG AGT GGG GAG AAC CTT CCT TAC CTG GTA GCG TAC CAA

                570                                                 580
 A  T  V  C  A  R  A  Q  A  P  P  P  S  W  D
GCC ACC GTG TGC GCT AGG GCT CAA GCC CCT CCC CCA TCG TGG GAC
```

FIG. 4C

```
                                                590
 Q   M   W   K   C   L   I   R   L   K   P   T   L   H   G
CAG ATG TGG AAG TGT TTG ATT CGC CTC AAG CCC ACC CTC CAT GGG

                600                                     610
 P   T   P   L   L   Y   R   L   G   A   V   Q   N   E   I
CCA ACA CCC CTG CTA TAC AGA CTG GGC GCT GTT CAG AAT GAA ATC


                                            620
 T   L   T   H   P   V   T   K   Y   I   M   T   C   M   S
ACC CTG ACG CAC CCA GTC ACC AAA TAC ATC ATG ACA TGC ATG TCG

                630                                     640
 A   D   L   E   V   V   T   S   T   W   V   L   V   G   G
GCC GAC CTG GAG GTC GTC ACG AGC ACC TGG GTG CTC GTT GGC GGC

                                            650
 V   L   A   A   L   A   A   Y   C   L   S   T   G   C   V
GTC CTG GCT GCT TTG GCC GCG TAT TGC CTG TCA ACA GGC TGC GTG

                660                                     670
 V   I   V   G   R   V   V   L   S   G   K   P   A   I   I
GTC ATA GTG GGC AGG GTC GTC TTG TCC GGG AAG CCG GCA ATC ATA

                                            680
 P   D   R   E   V   L   Y   R   E   F   D   E   M   E   E
CCT GAC AGG GAA GTC CTC TAC CGA GAG TTC GAT GAG ATG GAA GAG

686
 C
TGC
```

FIG. 4D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SalI
ClaI
1320bp
Ligate into pBS24.1 yeast expression vector
SalI
1320bp
ClaI
ClaI
683bp
BglII
ClaI
BglII
BglII +
HindIII
Ligate with oligos into pSP72 subcloning vector
pSP72 1aHC#1
HindIII
ClaI
ClaI +
761bp
HindIII
HindIII +
ADH2/GAPDH promoter
1353bp
BamHI
pd.HCV1a.ns3ns4aPI

FIG. 5

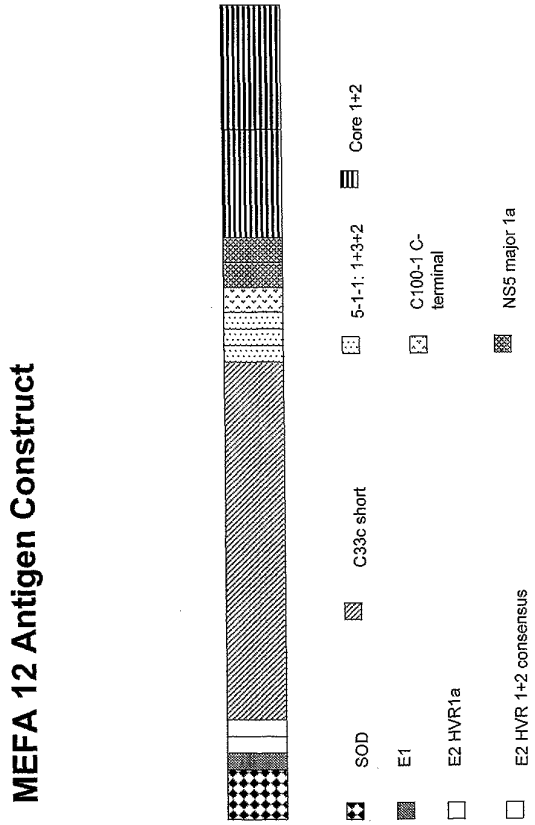
MEFA 12 Antigen Construct
SOD
C33c short
5-1-1: 1+3+2
Core 1+2
E1
E2 HVR1a
C100-1 C-terminal
E2 HVR 1+2 consensus
NS5 major 1a

FIG. 6

```
  1                                                  10
  M   A   T   K   A   V   C   V   L   K   G   D   G   P   V
ATG GCT ACA AAG GCT GTT TGT GTT TTG AAG GGT GAC GGC CCA GTT   45

                  20                                  30
  Q   G   I   I   N   F   E   Q   K   E   S   N   G   P   V
CAA GGT ATT ATT AAC TTC GAG CAG AAG GAA AGT AAT GGA CCA GTG   90

                                      40
  K   V   W   G   S   I   K   G   L   T   E   G   L   H   G
AAG GTG TGG GGA AGC ATT AAA GGA CTG ACT GAA GGC CTG CAT GGA  135

                  50                                  60
  F   H   V   H   E   F   G   D   N   T   A   G   C   T   S
TTC CAT GTT CAT GAG TTT GGA GAT AAT ACA GCA GGC TGT ACC AGT  180

                                      70
  A   G   P   H   F   N   P   L   S   T   R   G   C   N   C
GCA GGT CCT CAC TTT AAT CCT CTA TCC ACG CGT GGT TGC AAT TGC  225

                  80                                  90
  S   I   Y   P   G   H   I   T   G   H   R   M   A   W   K
TCT ATC TAT CCC GGC CAT ATA ACG GGT CAC CGC ATG GCA TGG AAG  270

                                     100
  L   G   S   A   A   R   T   T   S   G   F   V   S   L   F
CTT GGT TCC GCC GCC AGA ACT ACC TCG GGC TTT GTC TCC TTG TTC  315

                 110                                 120
  A   P   G   A   K   Q   N   E   T   H   V   T   G   G   A
GCC CCA GGT GCC AAA CAA AAC GAA ACT CAC GTC ACG GGA GGC GCA  360

                                     130
  A   A   R   T   T   S   G   L   T   S   L   F   S   P   G
GCC GCC CGA ACT ACG TCT GGG TTG ACC TCT TTG TTC TCC CCA GGT  405
```

FIG. 7A

```
                  140                                             150
 A   S   Q   N   I   Q   L   I   T   S   T   D   N   S   S
GCC AGC CAA AAC ATT CAA TTG ATT ACT AGT ACG GAT AAC TCC TCT  450

                                      160
 P   P   V   V   P   Q   S   F   Q   V   A   H   L   H   A
CCA CCA GTA GTG CCC CAG AGC TTC CAG GTG GCT CAC CTC CAT GCT  495

                  170                                             180
 P   T   G   S   G   K   S   T   K   V   P   A   A   Y   A
CCC ACA GGC AGC GGC AAA AGC ACC AAG GTC CCG GCT GCA TAT GCA  540

                                      190
 A   Q   G   Y   K   V   L   V   L   N   P   S   V   A   A
GCT CAG GGC TAT AAG GTG CTA GTA CTC AAC CCC TCT GTT GCT GCA  585

                  200                                             210
 T   L   G   F   G   A   Y   M   S   K   A   H   G   I   D
ACA CTG GGC TTT GGT GCT TAC ATG TCC AAG GCT CAT GGG ATC GAT  630

                                      220
 P   N   I   R   T   G   V   R   T   I   T   T   G   S   P
CCT AAC ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT GGC AGC CCC  675

                  230                                             240
 I   T   Y   S   T   Y   G   K   F   L   A   D   G   G   C
ATC ACG TAC TCC ACC TAC GGC AAG TTC CTT GCC GAC GGC GGG TGC  720

                                      250
 S   G   G   A   Y   D   I   I   I   C   D   E   C   H   S
TCG GGG GGC GCT TAT GAC ATA ATA ATT TGT GAC GAG TGC CAC TCC  765

                  260                                             270
 T   D   A   T   S   I   L   G   I   G   T   V   L   D   Q
ACG GAT GCC ACA TCC ATC TTG GGC ATC GGC ACT GTC CTT GAC CAA  810

                                      280
 A   E   T   A   G   A   R   L   V   V   L   A   T   A   T
GCA GAG ACT GCG GGG GCG AGA CTG GTT GTG CTC GCC ACC GCC ACC  855

                  290                                             300
 P   P   G   S   V   T   V   P   H   P   N   I   E   E   V
CCT CCG GGC TCC GTC ACT GTG CCC CAT CCC AAC ATC GAG GAG GTT  900
```

FIG. 7B

```
                                    310
 A   L   S   T   T   G   E   I   P   F   Y   G   K   A   I
GCT CTG TCC ACC ACC GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC  945

                320                                     330
 P   L   E   V   I   K   G   G   R   H   L   I   F   C   H
CCC CTC GAA GTA ATC AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT  990

                                    340
 S   K   K   K   C   D   E   L   A   A   K   L   V   A   L
TCA AAG AAG AAG TGC GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG 1035

                350                                     360
 G   I   N   A   V   A   Y   Y   R   G   L   D   V   S   V
GGC ATC AAT GCC GTG GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC 1080

                                    370
 I   P   T   S   G   D   V   V   V   V   A   T   D   A   L
ATC CCG ACC AGC GGC GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC 1125

                380                                     390
 M   T   G   Y   T   G   D   F   D   S   V   I   D   C   N
ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT 1170

                                    400
 T   C   A   C   S   G   K   P   A   I   I   P   D   R   E
ACG TGT GCA TGC TCC GGG AAG CCG GCA ATC ATA CCT GAC AGG GAA 1215

                410                                     420
 V   L   Y   R   E   F   D   E   M   E   E   C   S   Q   H
GTC CTC TAC CGA GAG TTC GAT GAG ATG GAA GAG TGC TCT CAG CAC 1260

                                    430
 L   P   Y   I   E   Q   G   M   M   L   A   E   Q   F   K
TTA CCG TAC ATC GAG CAA GGG ATG ATG CTC GCC GAG CAG TTC AAG 1305

                440                                     450
 Q   K   A   L   G   L   S   R   G   G   K   P   A   I   V
CAG AAG GCC CTC GGC CTC TCG CGA GGG GGC AAG CCG GCA ATC GTT 1350

                                    460
 P   D   K   E   V   L   Y   Q   Q   Y   D   E   M   E   E
CCA GAC AAA GAG GTG TTG TAT CAA CAA TAC GAT GAG ATG GAA GAG 1395
```

FIG. 7C

```
                    470                                                 480
 C   S   Q   A   A   P   Y   I   E   Q   A   Q   V   I   A
TGC TCA CAA GCT GCC CCA TAT ATC GAA CAA GCT CAG GTA ATA GCT  1440

                                    490
 H   Q   F   K   E   K   V   L   G   L   I   D   N   D   Q
CAC CAG TTC AAG GAA AAA GTC CTT GGA TTG ATC GAT AAT GAT CAA  1485

                    500                                                 510
 V   V   V   T   P   D   K   E   I   L   Y   E   A   F   D
GTG GTT GTG ACT CCT GAC AAA GAA ATC TTA TAT GAG GCC TTT GAT  1530

                                    520
 E   M   E   E   C   A   S   K   A   A   L   I   E   E   G
GAG ATG GAA GAA TGC GCC TCC AAA GCC GCC CTC ATT GAG GAA GGG  1575

                    530                                                 540
 Q   R   M   A   E   M   L   K   S   K   I   Q   G   L   L
CAG CGG ATG GCG GAG ATG CTC AAG TCT AAG ATA CAA GGC CTC CTC  1625

                                    550
 G   I   L   R   R   H   V   G   P   G   E   G   A   V   Q
GGG ATA CTG CGC CGG CAC GTT GGT CCT GGC GAG GGG GCA GTG CAG  1670

                    560                                                 570
 W   M   N   R   L   I   A   F   A   S   R   G   N   H   V
TGG ATG AAC CGG CTG ATA GCC TTC GCC TCC AGA GGG AAC CAT GTT  1715

                                    580
 S   P   T   H   Y   V   P   S   R   S   R   R   F   A   Q
TCC CCC ACG CAC TAC GTT CCG TCT AGA TCC CGG AGA TTC GCC CAG  1760

                    590                                                 600
 A   L   P   V   W   A   R   P   D   Y   N   P   P   L   V
GCC CTG CCC GTT TGG GCG CGG CCG GAC TAT AAC CCC CCG CTA GTG  1805

                                    610
 E   T   W   K   K   P   D   Y   E   P   P   V   V   H   G
GAG ACG TGG AAA AAG CCC GAC TAC GAA CCA CCT GTG GTC CAC GGC  1850

                    620                                                 630
 R   S   S   R   R   F   A   Q   A   L   P   V   W   A   R
AGA TCT TCT CGG AGA TTC GCC CAG GCC CTG CCC GTT TGG GCG CGG  1895
```

FIG. 7D

```
                                        640
 P   D   Y   N   P   P   L   V   E   T   W   K   K   P   D
CCG GAC TAT AAC CCC CCG CTA GTG GAG ACG TGG AAA AAG CCC GAC  1940

                650                                     660
 Y   E   P   P   V   V   H   G   R   K   T   K   R   N   T
TAC GAA CCA CCT GTG GTC CAT GGC AGA AAG ACC AAA CGT AAC ACC  1985

                                    670
 N   R   R   P   Q   D   V   K   F   P   G   G   G   Q   I
AAC CGG CGG CCG CAG GAC GTC AAG TTC CCG GGT GGC GGT CAG ATC  2030

                680                                     690
 V   G   G   V   Y   L   L   P   R   R   G   P   R   L   G
GTT GGT GGA GTT TAC TTG TTG CCG CGC AGG GGC CCT AGA TTG GGT  2075

                                    700
 V   L   A   T   R   K   T   S   P   I   P   K   A   R   R
GTG CTC GCG ACG AGA AAG ACT TCC CCT ATC CCC AAG GCT CGT CGG  2120

                710                                     720
 P   E   G   R   T   W   A   Q   P   G   Y   P   W   P   L
CCC GAG GGC AGG ACC TGG GCT CAG CCC GGT TAC CCT TGG CCC CTC  2165

                                    730
 Y   G   N   K   D   R   R   S   T   G   K   S   W   G   K
TAT GGC AAT AAG GAC AGA CGG TCT ACA GGT AAG TCC TGG GGT AAG  2210

                740                                     750
 P   G   Y   P   W   P   R   K   T   K   R   N   T   N   R
CCA GGG TAC CCT TGG CCA AGA AAG ACC AAA CGT AAC ACC AAC CGG  2255

                                    760
 R   P   Q   D   V   K   F   P   G   G   G   Q   I   V   G
CGG CCG CAG GAC GTC AAG TTC CCG GGT GGC GGT CAG ATC GTT GGT  2300

                770                                     780
 G   V   Y   L   L   P   R   R   G   P   R   L   G   V   L
GGA GTT TAC TTG TTG CCG CGC AGG GGC CCT AGA TTG GGT GTG CTC  2345

                                    790
 A   T   R   K   T   S   P   I   P   K   A   R   R   P   E
GCG ACG AGA AAG ACT TCC CCT ATC CCC AAG GCT CGT CGG CCC GAG  2390
```

FIG. 7E

```
                  800                                                 810
 G   R   T   W   A   Q   P   G   Y   P   W   P   L   Y   G
GGC AGG ACC TGG GCT CAG CCC GGT TAC CCT TGG CCC CTC TAT GGC-  2435

                                      820
 N   K   D   R   R   S   T   G   K   S   W   G   K   P   G
AAT AAG GAC AGA CGG TCT ACA GGT AAG TCC TGG GGT AAG CCA GGG  2480

              829
 Y   P   W   P   OC
TAC CCT TGG CCC TAA TGAGTCGAC
```

FIG. 7F

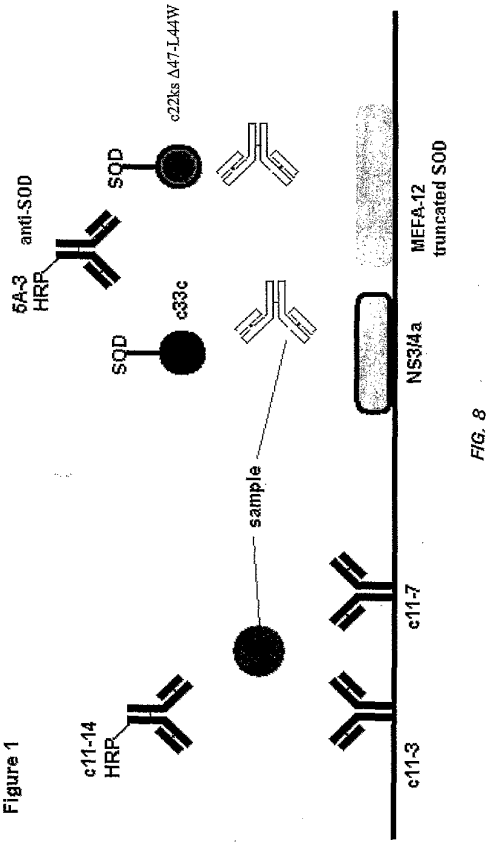

Figure 1

FIG. 8

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(54) Title: HCV ANTIGEN/ANTIBODY COMBINATION ASSAY

(57) Abstract: An HCV core antigen and NS3/4a antibody combination assay that can detect both HCV antigens and antibodies present in a sample using a single solid matrix, is provided, as well as immunoassay solid supports for use in the assay.

HCV ANTIGEN/ANTIBODY COMBINATION ASSAY

Technical Field

The present invention pertains generally to viral diagnostics. In particular, the invention relates to an antigen/antibody combination assay for accurately diagnosing hepatitis C virus infection.

Background Of The Invention

Hepatitis C Virus (HCV) is the principal cause of parenteral non-A, non-B hepatitis (NANBH) which is transmitted largely through blood transfusion and sexual contact. The virus is present in 0.4 to 2.0% of blood donors. Chronic hepatitis develops in about 50% of infections and of these, approximately 20% of infected individuals develop liver cirrhosis which sometimes leads to hepatocellular carcinoma. Accordingly, the study and control of the disease is of medical importance.

HCV was first identified and characterized as a cause of NANBH by Houghten et al. The viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. HCV has a 9.5 kb positive-sense, single-stranded RNA genome and is a member of the Flaviridae family of viruses. At least six distinct, but related genotypes of HCV, based on phylogenetic analyses, have been identified (Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399). The virus encodes a single polyprotein having more than 3000 amino acid residues (Choo et al., *Science* (1989) 244:359-362; Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

In particular, as shown in Figure 1, several proteins are encoded by the HCV genome. The order and nomenclature of the cleavage products of the HCV polyprotein is as follows: NH_2-C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. Initial cleavage of the polyprotein is catalyzed by host proteases which liberate three structural proteins, the N-terminal nucleocapsid protein (termed "core") and two envelope glycoproteins, "E1" (also known as E) and "E2" (also known as E2/NS1), as well as nonstructural (NS) proteins that contain the viral enzymes. The NS regions are termed NS2, NS3, NS4 and NS5. NS2 is an integral membrane protein with proteolytic activity. NS2, either alone or in combination with NS3, cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease serves to process the remaining polyprotein. Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease. Subsequent NS3-mediated cleavages of the HCV polyprotein appear to involve recognition of polyprotein cleavage junctions by an NS3 molecule of another polypeptide. In these reactions, NS3 liberates an NS3 cofactor (NS4a), two proteins (NS4b and NS5a), and an RNA-dependent RNA polymerase (NS5b).

A number of general and specific polypeptides useful as immunological and diagnostic reagents for HCV, derived from the HCV polyprotein, have been described. See, e.g., Houghton et al., European Publication Nos. 318,216 and 388,232; Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Houghton et al., *Hepatology* (1991) 14:381-388; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778. These publications provide an extensive background on HCV generally, as well as on the manufacture and uses of HCV polypeptide immunological reagents.

Sensitive, specific methods for screening and identifying carriers of HCV and HCV-contaminated blood or blood products would provide an important advance in medicine. Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfused patients, and HCV has accounted for up to 90% of these cases. Patient care as well as the

prevention and transmission of HCV by blood and blood products or by close personal contact require reliable diagnostic and prognostic tools. Accordingly, several assays have been developed for the serodiagnosis of HCV infection. See, e.g., Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Choo et al., *Br. Med. Bull.* (1990) 46:423-441; Ebeling et al., *Lancet* (1990) 335:982-983; van der Poel et al., *Lancet* (1990) 335:558-560; van der Poel et al., *Lancet* (1991) 337:317-319; Chien, D.Y., International Publication No. WO 94/01778; Valenzuela et al., International Publication No. WO 97/44469; and Kashiwakuma et al., U.S. Patent No. 5,871,904.

A significant problem encountered with some serum-based assays is that there is a significant gap between infection and detection of the virus, often exceeding 80 days. This assay gap may create great risk for blood transfusion recipients. To overcome this problem, nucleic acid-based tests (NAT) that detect viral RNA directly, and HCV core antigen tests that assay viral antigen instead of antibody response, have been developed. See, e.g., Kashiwakuma et al., U.S. Patent No. 5,871,904; Beld et al., *Transfusion* (2000) 40:575-579.

However, there remains a need for sensitive, accurate diagnostic and prognostic tools in order to provide adequate patient care as well as to prevent transmission of HCV by blood and blood products or by close personal contact.

Summary of the Invention

The present invention is based in part, on the finding that HCV seroconversion antibodies are typically anti-core and anti-NS3 (helicase). Accordingly, the invention provides an HCV core antigen and NS3 antibody combination assay that can detect both HCV antigens and antibodies present in a sample using a single solid matrix.

Thus, in one embodiment, the subject invention is directed to an immunoassay solid support comprising at least one HCV anti-core antibody and at least one isolated HCV NS3/4a epitope bound thereto. The antibody and NS3/4a epitope can be any of the herein described molecules. Additionally, the solid support may include any of the multiple epitope fusion antigens described herein, such as the multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F.

In certain embodiments, the solid support comprises at least two HCV anti-core antibodies bound thereto. Moreover, the anti-core antibody may be a monoclonal antibody. Additionally, the NS3/4a epitope may be a conformational epitope, such as a conformational NS3/4a epitope comprising the amino acid sequence depicted in Figures 4A-4D.

In another embodiment, the invention is directed to an immunoassay solid support comprising at least two HCV anti-core monoclonal antibodies and at least one HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, bound thereto.

In still a further embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support as described above; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least one anti-core antibody and the NS3/4a epitope, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) an antigen that reacts with an HCV antibody from the biological sample reactive with the NS3/4a epitope; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the antigen of (ii); and (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample. The NS3/4a epitope may be a conformational epitope, such as a conformational epitope having the NS3/4a sequence depicted in Figures 4A-4D.

In yet another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support with at least two HCV anti-core antibodies bound thereto, as described above; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological

sample, to bind to the at least two anti-core antibodies and the NS3/4a epitope, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the hSOD amino acid sequence; and (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample. The NS3/4a epitope may be a conformational epitope, such as a conformational epitope having the NS3/4a sequence depicted in Figures 4A-4D.

In any of the above embodiments, the anti-core antibody may be directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence, and/or the detectably labeled HCV anti-core antibody may be directed against a C-terminal region of the HCV core antigen, such as amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence. Moreover, the antigen that reacts with an HCV antibody from the biological sample may be from the NS3 region, such as an epitope from the c33c region of the HCV polyprotein and can be fused with a human superoxide dismutase (hSOD) amino acid sequence. In this embodiment, the second detectably labeled antibody is reactive with the hSOD amino acid sequence.

In another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support including two HCV anti-core monoclonal antibodies and a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least two anti-core antibodies and the NS3/4a conformational epitope, respectively; adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled

antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with said hSOD amino acid sequence; detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

In certain embodiments, the at least two anti-core antibodies are directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein, and the detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen, such as against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

In another embodiment, the invention is directed to a method of detecting HCV infection in a biological sample. The method comprises: (a) providing an immunoassay solid support which includes a multiple epitope fusion antigen; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least one anti-core antibody, the NS3/4a epitope, and the multiple epitope fusion antigen; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) first and second antigens that react with an HCV antibody from the biological sample reactive with the NS3/4a epitope and the multiple epitope fusion antigen, respectively; and (iii) a second detectably labeled antibody, wherein the second detectably labeled antibody is reactive with the antigens of (ii); (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

The anti-core antibody may be directed against an N-terminal region of the HCV core antigen and said first detectably labeled HCV anti-core antibody may be directed against a C-terminal region of the HCV core antigen, as described above. Moreover, the first antigen that reacts with an HCV antibody from the biological sample may comprise an epitope from the c33c region of the HCV polyprotein, and may be fused with an hSOD amino acid sequence. In this context, the second detectably labeled antibody is reactive with the hSOD amino acid sequence. Additionally, the second antigen that reacts with an HCV antibody from the biological sample may comprise an epitope from the c22 region of the HCV polyprotein, such as an epitope comprising amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence. The epitope may be fused with an hSOD amino acid sequence. If so, the second detectably labeled antibody is reactive with the hSOD amino acid sequence. The multiple epitope fusion antigen may comprise the amino acid sequence depicted in Figures 7A-7F.

In yet a further embodiment, the invention is directed to a method of detecting HCV infection in a biological sample, said method comprising: (a) providing an immunoassay solid support which comprises two HCV anti-core monoclonal antibodies, an HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, and a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, bound thereto; (b) combining a biological sample with the solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the at least two anti-core antibodies, the NS3/4a conformational epitope, and the multiple epitope fusion antigen, respectively; (c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein the first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein the labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence and an epitope from the c22 region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody,

wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequences; (d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

In this embodiment, the at least two anti-core antibodies may be directed against an N-terminal region of the HCV core antigen, such as against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein, and the detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen, such as against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence. Moreover, the epitope from the c22 region may comprise amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence.

In other embodiments, the invention is directed to immunodiagnostic test kits comprising the immunoassay solid support described above, and instructions for conducting the immunodiagnostic test.

In still further embodiments, the invention is directed to methods of producing an immunoassay solid support, comprising: (a) providing a solid support; and (b) binding at least one HCV anti-core antibody, such as one or two or more, and at least one isolated HCV NS3/4a epitope thereto, and optionally, a multiple epitope fusion antigen thereto. The anti-core antibodies, NS3/4a epitopes and multiple epitope fusion antigens are as described above.

In additional embodiments, the invention is directed to a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 80% sequence identity, such as 90% or more sequence identity, thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual. In certain embodiments, the multiple epitope fusion antigen consists of the amino acid sequence depicted in Figures 5A-5F.

In further embodiments, the invention is directed to a polynucleotide comprising a coding sequence for the multiple epitope fusion antigen above, a recombinant vectors comprising the polynucleotides, host cells transformed with the recombinant vectors, and methods of producing a recombinant multiple epitope fusion antigen comprising:

(a) providing a population of host cells as above; and (b) culturing the population of cells under conditions whereby the multiple epitope fusion antigen encoded by the coding sequence present in the recombinant vector is expressed.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the HCV genome, depicting the various regions of the polyprotein from which the present assay reagents (proteins and antibodies) are derived.

Figure 2 is a schematic drawing of a representative antibody/antigen combination assay under the invention.

Figure 3 depicts the amino acid sequence of a representative NS3/4a conformational antigen for use in the present assays. The bolded alanine at position 182 is substituted for the native serine normally present at this position.

Figures 4A through 4D depict the DNA and corresponding amino acid sequence of another representative NS3/4a conformational antigen for use in the present assays. The amino acids at positions 403 and 404 of Figures 4A through 4D represent substitutions of Pro for Thr, and Ile for Ser, of the native amino acid sequence of HCV-1.

Figure 5 is a diagram of the construction of pd.HCV1a.ns3ns4aPI.

Figure 6 is a diagrammatic representation of MEFA 12.

Figures 7A-7F depict the DNA and corresponding amino acid sequence of MEFA 12.

Figure 8 is a schematic drawing of a representative immunoassay under the invention, using MEFA 12.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the

literature. See, e.g., *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more antigens, and the like.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length

proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

An HCV polypeptide is a polypeptide, as defined above, derived from the HCV polyprotein. The polypeptide need not be physically derived from HCV, but may be synthetically or recombinantly produced. Moreover, the polypeptide may be derived from any of the various HCV strains, such as from strains 1, 2, 3 or 4 of HCV. A number of conserved and variable regions are known between these strains and, in general, the amino acid sequences of epitopes derived from these regions will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned. Thus, for example, the term "NS3/4a" polypeptide refers to native NS3/4a from any of the various HCV strains, as well as NS3/4a analogs, muteins and immunogenic fragments, as defined further below. The complete genotypes of many of these strains are known. See, e.g., U.S. Patent No. 6,150,087 and GenBank Accession Nos. AJ238800 and AJ238799.

The terms "analog" and "mutein" refer to biologically active derivatives of the reference molecule, or fragments of such derivatives, that retain desired activity, such as immunoreactivity in the assays described herein. In general, the term "analog" refers to compounds having a native polypeptide sequence and structure with one or more amino acid additions, substitutions (generally conservative in nature) and/or deletions, relative to the native molecule, so long as the modifications do not destroy immunogenic activity. The term "mutein" refers to peptides having one or more peptide mimics ("peptoids"), such as those described in International Publication No. WO 91/04282. Preferably, the analog or mutein has at least the same immunoactivity as the native molecule. Methods

for making polypeptide analogs and muteins are known in the art and are described further below.

Particularly preferred analogs include substitutions that are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. For example, the polypeptide of interest may include up to about 5-10 conservative or non-conservative amino acid substitutions, or even up to about 15-25 conservative or non-conservative amino acid substitutions, or any integer between 5-25, so long as the desired function of the molecule remains intact. One of skill in the art may readily determine regions of the molecule of interest that can tolerate change by reference to Hopp/Woods and Kyte-Doolittle plots, well known in the art.

By "fragment" is intended a polypeptide consisting of only a part of the intact full-length polypeptide sequence and structure. The fragment can include a C-terminal deletion and/or an N-terminal deletion of the native polypeptide. An "immunogenic fragment" of a particular HCV protein will generally include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably at least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between 5 amino acids and the full-length sequence, provided that the fragment in question retains immunoreactivity in the assays described herein. For example, preferred immunogenic fragments, include but are not limited to fragments of HCV core that comprise, e.g., amino acids 10-45, 10-53, 67-88, and 120-

130 of the polyprotein, epitope 5-1-1 (in the NS3 region of the viral genome) as well as defined epitopes derived from the E1, E2, c33c (NS3), c100 (NS4), NS3/4a and NS5 regions of the HCV polyprotein, as well as any of the other various epitopes identified from the HCV polyprotein. See, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; U.S. Patent Nos. 6,150,087 and 6,121,020.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, binds to an antibody generated in response to such sequence. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes from the HCV polyprotein. An epitope for use in the subject invention is not limited to a polypeptide having the exact sequence of the portion of the parent protein from which it is derived. Indeed, viral genomes are in a state of constant flux and contain several variable domains which exhibit relatively high degrees of variability between isolates. Thus the term "epitope" encompasses sequences identical to the native sequence, as well as modifications to the native sequence, such as deletions, additions and substitutions (generally conservative in nature).

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:178-182; Geysen et al. (1986) *Molec. Immunol.* 23:709-715.

Using such techniques, a number of epitopes of HCV have been identified. See, e.g., Chien et al., *Viral Hepatitis and Liver Disease* (1994) pp. 320-324, and further below. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra.* Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omiga version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

As used herein, the term "conformational epitope" refers to a portion of a full-length protein, or an analog or mutein thereof, having structural features native to the amino acid sequence encoding the epitope within the full-length natural protein. Native structural features include, but are not limited to, glycosylation and three dimensional structure. The length of the epitope defining sequence can be subject to wide variations as these epitopes are believed to be formed by the three-dimensional shape of the antigen (e.g., folding). Thus, amino acids defining the epitope can be relatively few in number, but widely dispersed along the length of the molecule (or even on different molecules in the case of dimers, etc.), being brought into correct epitope conformation via folding. The portions of the antigen between the residues defining the epitope may not be critical to the conformational structure of the epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g., cysteines involved in disulfide bonding, glycosylation sites, etc.).

Conformational epitopes present in the NS3/4a region are readily identified using methods discussed above. Moreover, the presence or absence of a conformational epitope in a given polypeptide can be readily determined through screening the antigen of interest with an antibody (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains

only linear epitopes (if any). In such screening using polyclonal antibodies, it may be advantageous to absorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest. Additionally, in the case of NS3/4a, a molecule which preserves the native conformation will also have protease and, optionally, helicase enzymatic activities. Such activities can be detected using enzymatic assays, as described further below.

Preferably, a conformational epitope is produced recombinantly and is expressed in a cell from which it is extractable under conditions which preserve its desired structural features, e.g. without denaturation of the epitope. Such cells include bacteria, yeast, insect, and mammalian cells. Expression and isolation of recombinant conformational epitopes from the HCV polyprotein are described in e.g., International Publication Nos. WO 96/04301, WO 94/01778, WO 95/33053, WO 92/08734. Alternatively, it is possible to express the antigens and further renature the protein after recovery. It is also understood that chemical synthesis may also provide conformational antigen mimitopes that cross-react with the "native" antigen's conformational epitope.

The term "multiple epitope fusion antigen" or "MEFA" as used herein intends a polypeptide in which multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or may be separated by intervening amino acid sequences. The fusion antigens may also contain sequences exogenous to the HCV polyprotein. Moreover, the HCV sequences present may be from multiple genotypes and/or isolates of HCV. Examples of particular MEFAs for use in the present immunoassays are detailed in, e.g., International Publication No. WO 97/44469, and are described further below.

An "antibody" intends a molecule that, through chemical or physical means, specifically binds to a polypeptide of interest. Thus, an HCV core antibody is a molecule that specifically binds to the HCV core protein. The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Patent No. 4,816,567); F(ab')2 and F(ab)

fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar et al. (1972) *Proc Natl Acad Sci USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem* 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) *Proc Natl Acad Sci USA* 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. Thus, the term encompasses antibodies obtained from murine hybridomas, as well as human monoclonal antibodies obtained using human rather than murine hybridomas. See, e.g., Cote, et al. *Monclonal Antibodies and Cancer Therapy*, Alan R. Liss, 1985, p. 77.

A "recombinant" protein is a protein which retains the desired activity and which has been prepared by recombinant DNA techniques as described herein. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macromolecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

By "equivalent antigenic determinant" is meant an antigenic determinant from different sub-species or strains of HCV, such as from strains 1, 2, or 3 of HCV. More specifically, epitopes are known, such as 5-1-1, and such epitopes vary between the strains 1, 2, and 3. Thus, the epitope 5-1-1 from the three different strains are equivalent antigenic determinants and thus are "copies" even though their sequences are not identical. In general the amino acid sequences of equivalent antigenic determinants will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned.

"Homology" refers to the percent similarity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence similarity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100.

Readily available computer programs can be used to aid in the analysis of similarity and identity, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence similarity and identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the

default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent similarity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Another method of establishing percent similarity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence similarity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the

case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper transcription factors, etc., are present. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence, as can transcribed introns, and the promoter sequence can still be considered "operably linked" to the coding sequence.

A "control element" refers to a polynucleotide sequence which aids in the expression of a coding sequence to which it is linked. The term includes promoters, transcription termination sequences, upstream regulatory domains, polyadenylation signals, untranslated regions, including 5'-UTRs and 3'-UTRs and when appropriate, leader sequences and enhancers, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A "promoter" as used herein is a DNA regulatory region capable of binding RNA polymerase in a host cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence

into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

"Expression cassette" or "expression construct" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette includes control elements, as described above, such as a promoter which is operably linked to (so as to direct transcription of) the sequence(s) or gene(s) of interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the expression cassette described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (e.g., a M13 origin of replication), at least one multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

"Transformation," as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

"Common solid support" intends a single solid matrix to which the HCV polypeptides used in the subject immunoassays are bound covalently or by noncovalent means such as hydrophobic adsorption.

"Immunologically reactive" means that the antigen in question will react specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

"Immune complex" intends the combination formed when an antibody binds to an epitope on an antigen.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, chromophores, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin, strepavidin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include, but are not limited to, horse radish peroxidase (HRP), fluorescein, FITC, rhodamine, dansyl, umbelliferone, dimethyl acridinium ester (DMAE), Texas red, luminol, NADPH and α-β-galactosidase.

II. Modes of Carrying out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

As noted above, the present invention is based on the discovery of novel diagnostic methods for accurately detecting early HCV infection. The methods rely on the identification and use of highly immunogenic HCV antibodies and antigens which are

present during the early stages of HCV seroconversion, thereby increasing detection accuracy and reducing the incidence of false results. The methods can be conveniently practiced in a single assay format.

More particularly, the assay is conducted on a solid support to which has been bound one or more HCV anti-core antibodies (directed against either the same or different HCV core epitopes) and an epitope derived from the NS3/4a region of the HCV polyprotein. Examples of particular anti-core antibodies useful in the present invention include, but are not limited to, antibody molecules such as monoclonal antibodies, directed against epitopes in the core region found between amino acids 10-53; amino acids 10-45; amino acids 67-88; amino acids 120-130, or antibodies directed against any of the core epitopes identified in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818.

The NS3/4a region of the HCV polyprotein has been described and the amino acid sequence and overall structure of the protein are disclosed in, e.g., Yao et al., *Structure* (November 1999) 7:1353-1363; Sali et al., *Biochem.* (1998) 37:3392-3401; and Bartenschlager, R., *J. Viral Hepat.* (1999) 6:165-181. See, also, Dasmahapatra et al., U.S. Patent No. 5,843,752. The subject immunoassays utilize at least one conformational epitope derived from the NS3/4a region that exists in the conformation as found in the naturally occurring HCV particle or its infective product, as evidenced by the preservation of protease and, optionally, helicase enzymatic activities normally displayed by the NS3/4a gene product and/or immunoreactivity of the antigen with antibodies in a biological sample from an HCV-infected subject, and a loss of the epitope's immunoreactivity upon denaturation of the antigen. For example, the conformational epitope can be disrupted by heating, changing the pH to extremely acid or basic, or by adding known organic denaturants, such as dithiothreitol (DTT) or an appropriate detergent. See, e.g., *Protein Purification Methods, a practical approach* (E.L.V. Harris

and S. Angal eds., IRL Press) and the denatured product compared to the product which is not treated as above.

Protease and helicase activity may be determined using standard enzyme assays well known in the art. For example, protease activity may be determined using assays well known in the art. See, e.g., Takeshita et al., *Anal. Biochem.* (1997) 247:242-246; Kakiuchi et al., *J. Biochem.* (1997) 122:749-755; Sali et al., *Biochemistry* (1998) 37:3392-3401; Cho et al., *J. Virol. Meth.* (1998) 72:109-115; Cerretani et al., *Anal. Biochem.* (1999) 266:192-197; Zhang et al., *Anal. Biochem.* (1999) 270:268-275; Kakiuchi et al., *J. Virol. Meth.* (1999) 80:77-84; Fowler et al., *J. Biomol. Screen.* (2000) 5:153-158; and Kim et al., *Anal. Biochem.* (2000) 284:42-48. A particularly convenient assay for testing protease activity is set forth in the examples below.

Similarly, helicase activity assays are well known in the art and helicase activity of an NS3/4a epitope may be determined using, for example, an ELISA assay, as described in, e.g., Hsu et al., *Biochem. Biophys. Res. Commun.* (1998) 253:594-599; a scintillation proximity assay system, as described in Kyono et al., *Anal. Biochem.* (1998) 257:120-126; high throughput screening assays as described in, e.g., Hicham et al., *Antiviral Res.* (2000) 46:181-193 and Kwong et al., *Methods Mol. Med.* (2000) 24:97-116; as well as by other assay methods known in the art. See, e.g., Khu et al., *J. Virol.* (2001) 75:205-214; Utama et al., *Virology* (2000) 273:316-324; Paolini et al., *J. Gen. Virol.* (2000) 81:1335-1345; Preugschat et al., *Biochemistry* (2000) 39:5174-5183; Preugschat et al., *Methods Mol. Med.* (1998) 19:353-364; and Hesson et al., *Biochemistry* (2000) 39:2619-2625.

The length of the antigen is sufficient to maintain an immunoreactive conformational epitope. Often, the polypeptide containing the antigen used will be almost full-length, however, the polypeptide may also be truncated to, for example, increase solubility or to improve secretion. Generally, the conformational epitope found in NS3/4a is expressed as a recombinant polypeptide in a cell and this polypeptide provides the epitope in a desired form, as described in detail below.

Representative amino acid sequences for NS3/4a polypeptides are shown in Figure 3 and Figures 4A through 4D. The bolded alanine occurring at position 182 of

Figure 3 is substituted for the native serine found at this position in order to prevent autocatalyisis of the molecule that might otherwise occur. The amino acid sequence shown at positions 2-686 of Figures 4A through 4D corresponds to amino acid positions 1027-1711 of HCV-1. An initiator codon (ATG) coding for Met, is shown as position 1. Additionally, the Thr normally occurring at position 1428 of HCV-1 (amino acid position 403 of Figure 4) is mutated to Pro, and the Ser normally occurring at position 1429 of HCV-1 (amino acid position 404 of Figure 4) is mutated to Ile. However, either the native sequence, with or without an N-terminal Met, the depicted analog, with or without the N-terminal Met, or other analogs and fragments can be used in the subject assays, so long as the epitope is produced using a method that retains or reinstates its native conformation such that protease activity, and optionally, helicase activity is retained. Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276, both describe analogs of NS3/4a.

The NS3 protease of NS3/4a is found at about positions 1027-1207, numbered relative to HCV-1, positions 2-182 of Figure 4. The structure of the NS3 protease and active site are known. See, e.g., De Francesco et al., *Antivir. Ther.* (1998) 3:99-109; Koch et al., *Biochemistry* (2001) 40:631-640. Changes to the native sequence that will normally be tolerated will be those outside of the active site of the molecule. Particularly, it is desirable to maintain amino acids 1- or 2-155 of Figure 4, with little or only conservative substitutions. Amino acids occurring beyond 155 will tolerate greater changes. Additionally, if fragments of the NS3/4a sequence found in Figure 4 are used, these fragments will generally include at least amino acids 1- or 2-155, preferably amino acids 1- or 2-175, and most preferably amino acids 1- or 2-182, with or without the N-terminal Met. The helicase domain is found at about positions 1193-1657 of HCV-1 (positions 207-632 of Figure 4). Thus, if helicase activity is desired, this portion of the molecule will be maintained with little or only conservative changes. One of skill in the art can readily determine other regions that will tolerate change based on the known structure of NS3/4a.

The solid support may also comprise other antigens. For example, multiple epitope fusion antigens (termed "MEFAs"), as described in International Publication No.

WO 97/44469, may be bound to the solid support for use in the subject assays. Such MEFAs include multiple epitopes derived from two or more of the various viral regions shown in Figure 1 and Table 1. In particular, as shown in Figure 1 and Table 1, An HCV polyprotein, upon cleavage, produces at least ten distinct products, in the order of NH_2-Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. The core polypeptide occurs at positions 1-191, numbered relative to HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, for the HCV-1 genome). This polypeptide is further processed to produce an HCV polypeptide with approximately amino acids 1-173. The envelope polypeptides, E1 and E2, occur at about positions 192-383 and 384-746, respectively. The P7 domain is found at about positions 747-809. NS2 is an integral membrane protein with proteolytic activity and is found at about positions 810-1026 of the polyprotein. NS2, either alone or in combination with NS3 (found at about positions 1027-1657), cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease, found at about positions 1027-1207, serves to process the remaining polyprotein. The helicase activity is found at about positions 1193-1657. Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease. Subsequent NS3-mediated cleavages of the HCV polyprotein appear to involve recognition of polyprotein cleavage junctions by an NS3 molecule of another polypeptide. In these reactions, NS3 liberates an NS3 cofactor (NS4a, found about positions 1658-1711), two proteins (NS4b found at about positions 1712-1972, and NS5a found at about positions 1973-2420), and an RNA-dependent RNA polymerase (NS5b found at about positions 2421-3011).

Table 1	
Domain	Approximate Boundaries*
C (core)	1-191
E1	192-383
E2	384-746
P7	747-809
NS2	810-1026
NS3	1027-1657
NS4a	1658-1711
NS4b	1712-1972
NS5a	1973-2420
NS5b	2421-3011

*Numbered relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455.

The multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. Thus, the linear order of the epitopes is different than their linear order in the genome in which they occur. The linear order of the sequences of the MEFAs for use herein is preferably arranged for optimum antigenicity. Preferably, the epitopes are from more than one HCV strain, thus providing the added ability to detect multiple strains of HCV in a single assay. Thus, the MEFAs for use herein may comprise various immunogenic regions derived from the polyprotein described above. Moreover, a protein resulting from a frameshift in the core region of the polyprotein, such as described in International Publication No. WO 99/63941, may be used in the MEFAs. If desired, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more of one or more epitopes derived from the HCV polyprotein may occur in the fusion protein.

For example, epitopes derived from, e.g., the hypervariable region of E2, such as a region spanning amino acids 384-410 or 390-410, can be included in the MEFA antigen. A particularly effective E2 epitope is one which includes a consensus sequence derived from this region, such as the consensus sequence Gly-Ser-Ala-Ala-Arg-Thr-Thr-Ser-Gly-Phe-Val-Ser-Leu-Phe-Ala-Pro-Gly-Ala-Lys-Gln-Asn, which represents a consensus sequence for amino acids 390-410 of the HCV type 1 genome. A representative E2 epitope present in a MEFA of the invention can comprise a hybrid epitope spanning amino acids 390-444. Such a hybrid E2 epitope can include a consensus sequence representing amino acids 390-410 fused to the native amino acid sequence for amino acids 411-444 of HCV E2.

Additionally, the antigens may be derived from various HCV strains. Multiple viral strains of HCV are known, and epitopes derived from any of these strains can be used in a fusion protein. It is well known that any given species of organism varies from one individual organism to another and further that a given organism such as a virus can have a number of different strains. For example, as explained above, HCV includes at least 6 genotypes. Each of these genotypes includes equivalent antigenic determinants. More specifically, each strain includes a number of antigenic determinants that are present on all strains of the virus but are slightly different from one viral strain to another. For example, HCV includes the antigenic determinant known as 5-1-1 (See, Figure 1). This particular antigenic determinant appears in three different forms on the three different viral strains of HCV. Accordingly, in a preferred embodiment of the invention all three forms of 5-1-1 appear on the multiple epitope fusion antigen used in the subject immunoassays. Similarly, equivalent antigenic determinants from the core region of different HCV strains may also be present. In general, equivalent antigenic determinants have a high degree of homology in terms of amino acid sequence which degree of homology is generally 30% or more, preferably 40% or more, when aligned. The multiple copy epitope of the present invention can also include multiple copies which are exact copies of the same epitope.

Representative MEFAs for use with the present assays are described in International Publication No. WO 97/44469. Additional representative MEFAs for use

herein include those termed MEFA 12, MEFA 13 and MEFA 13.1. It is to be understood that these MEFAs are merely representative and other epitopes derived from the HCV genome will also find use with the present assays and may be incorporated into these or other MEFAs.

The DNA sequence and corresponding amino acid sequence of MEFA 12 is shown in Figures 7A through 7F. The general structural formula for MEFA 12 is shown in Figure 6 and is as follows: hSOD-E1(type 1)-E2 HVR consensus(type 1a)-E2 HVR consensus(types 1 and 2)-c33c short(type 1)-5-1-1(type 1)-5-1-1(type 3)-5-1-1(type 2)-c100(type 1)-NS5(type 1)-NS5(type 1)-core(types 1+2)-core(types 1+2). This multiple copy epitope includes the following amino acid sequence, numbered relative to HCV-1 (the numbering of the amino acids set forth below follows the numbering designation provided in Choo, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, in which amino acid #1 is the first methionine encoded by the coding sequence of the core region): amino acids 1-69 of superoxide dismutase (SOD, used to enhance recombinant expression of the protein); amino acids 303 to 320 of the polyprotein from the E1 region; amino acids 390 to 410 of the polyprotein, representing a consensus sequence for the hypervariable region of HCV-1a E2; amino acids 384 to 414 of the polyprotein from region E2, representing a consensus sequence for the E2 hypervariable regions of HCV-1 and HCV-2; amino acids 1211-1457 of the HCV-1 polyprotein which define the helicase; three copies of an epitope from 5-1-1, amino acids 1689-1735, one from HCV-1, one from HCV-3 and one from HCV-2, which copies are equivalent antigenic determinants from the three different viral strains of HCV; HCV polypeptide C100 of HCV-1, amino acids 1901-1936 of the polyprotein; two exact copies of an epitope from the NS5 region of HCV-1, each with amino acids 2278 to 2313 of the HCV polyprotein; and two copies of three epitopes from the core region, two from HCV-1 and one from HCV-2, which copies are equivalent antigenic determinants represented by amino acids 9 to 53 and 64-88 of HCV-1 and 67-84 of HCV-2.

Table 2 shows the amino acid positions of the various epitopes in MEFA 12 with reference to Figures 7A through 7F herein. The numbering in the tables is relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455. MEFAs 13

and 13.1 also share the general formula specified above for MEFA 12, with modifications as indicated in Tables 3 and 4, respectively.

Table 2. MEFA 12

mefa aa#	5' end site	epitope	hcv aa#	strain
1-69*	*Nco*1	hSOD		
72-89	*Mlu*I	E1	303-320	1
92-112	*Hind*111	E2 HVR1a consensus	390-410	1
113-143		E2 HVR1+2 consensus	384-414	1, 2
146-392	*Spe*I	C33C short	1211-1457	1
395-441	*Sph*I	5-1-1	1689-1735	1
444-490	*Nru*I	5-1-1	1689-1735	3
493-539	*Cla*I	5-1-1	1689-1735	2
542-577	*Ava*I	C100	1901-1936	1
580-615	*Xba*I	NS5	2278-2313	1
618-653	*Bgl*II	NS5	2278-2313	1
654-741	*Nco*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2
742-829	*Bal*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The SOD protein is truncated so that so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

Table 3. MEFA 13

mefa aa#	5' end site	epitope	hcv aa#	strain
1-156	*Nco*1	mutated hSOD (aa 70-72, ALA)		
161-178	*Mlu*I	E1	303-320	1
181-201	*Hind*111	E2 HVR1a consensus	390-410	1
202-232		E2 HVR1+2 consensus	384-414	1, 2
235-451		C33C short	1211-1457	1
454-500	*Hind*III	5-1-1 PImut*	1689-1735	1
503-549	*Nru*I	5-1-1 PImut*	1689-1735	3
552-598	*Cla*I	5-1-1 PImut*	1689-1735	2
601-636	*Ava*I	C100	1901-1936	1
639-674	*Xba*I	NS5	2278-2313	1
677-712	*Bgl*II	NS5	2278-2313	1
713-800		core epitopes	9-53, R47L 64-88 67-84	1 1 2
801-888		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI. Additionally, the SOD protein is mutated so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

Table 4. MEFA 13.1

mefa aa#	5' end site	epitope	hcv aa#	strain
1-86	*Nco*I	mutated hSOD (aa 70-72, ALA)		
89-106	*Mlu*I	E1	303-320	1
109-129	*Hind*III	E2 HVR1a consensus	390-410	1
130-160		E2 HVR1+2 consensus	384-414	1, 2
163-379		C33C short	1211-1457	1
382-428	*Hind*III	5-1-1 PImut*	1689-1735	1
431-477	*Nru*I	5-1-1 PImut*	1689-1735	3
480-526	*Cla*I	5-1-1 PImut*	1689-1735	2
529-564	*Ava*I	C100	1901-1936	1
567-602	*Xba*I	NS5	2278-2313	1
605-640	*Bgl*II	NS5	2278-2313	1
641-728		core epitopes	9-53, R47L 64-88 67-84	1 1 2
729-816		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI. Additionally, the SOD protein is mutated so that the detection conjugate, an HRP-labeled anti-SOD antibody does not bind the MEFA. The core epitope is mutated to prevent the antibodies to HCV core, used in detection, from binding to the MEFA.

In one assay format, the sample is combined with the solid support, as described further below. If the sample is infected with HCV, core antigens, as well as HCV

antibodies to those epitopes present on the solid support, will bind to the solid support components. A detectably labeled anti-core antibody is then added. The labeled anti-core antibody is directed against a different epitope than the anti-core antibody that is bound to the solid support. This anti-core antibody binds the core antigen captured by the anti-core antibodies on the solid support.

An antigen that reacts with the captured HCV antibody from the biological sample, which captured sample HCV antibody is reactive with the NS3/4a epitope, is also added. This antigen is preferably an epitope derived from the NS3 region of the HCV polyprotein. This antigen binds the captured HCV antibody from the sample. A number of antigens including such epitopes are known, including, but not limited to antigens derived from the c33c and c100 regions, as well as fusion proteins comprising an NS3 epitope, such as c25. These and other NS3 epitopes are useful in the present assays and are known in the art and described in, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818.

A second labeled antibody, directed against the antigen described above, is added. This antibody can be directed against any epitope included in the antigen. For example, the antibody can be directed against the NS3 region present in the antigen. Alternatively, if the antigen above is expressed as a fusion protein, the second labeled antibody can be directed against the fusion partner. Additional antigens and antibodies can be added to the assay, particularly if the solid support includes a MEFA. These assay formats are explained further below.

A representative assay under the invention is depicted in Figure 2. As shown in the figure, the solid support includes two anti-core monoclonal antibodies, termed c11-3 and c11-7. These antibodies are directed against an epitope found in the N-terminal region of the core protein at amino acids 10-53, numbered relative to the HCV1 polyprotein sequence. The solid support also includes an epitope to NS3/4a. The biological sample is added to the solid support. HCV core antigen, as well as antibodies

directed against the NS3/4a epitope, both present in the sample, will bind the capture reagents on the solid support.

Horse radish peroxidase (HRP)-labeled anti-core monoclonal antibody c11-14, directed against a C-terminal region of the core found at amino acid positions 120-130, numbered relative to the HCV1 polyprotein sequence, is then added. A fusion protein, comprising a sequence from human SOD (hSOD) and an epitope from the c33c region is added, as is a second HRP-labeled antibody, directed against the SOD portion of the fusion protein. The SOD-c33c fusion will bind to the anti-NS3 antibody and the anti-SOD antibody will, in turn, bind the SOD-c33c fusion protein. Detection of the label indicates the presence of HCV infection.

Another representative assay under the invention is depicted in Figure 8. The antibody assay configuration is an antigen-antibody-antigen sandwich capture assay using both NS3/4a and MEFA 12. The solid support includes the two anti-core monoclonal antibodies described above, an epitope to NS3/4a, as well as a representative MEFA, MEFA 12, which includes a truncated version of human SOD. As with the assay above, the biological sample is added to the solid support. HCV core antigen, as well as antibodies directed against the NS3/4a epitope and epitopes of the MEFA, present in the sample, will bind the capture reagents on the solid support. Two antigens, one reactive with sample antibodies that bind NS3/4a (as described above) and one reactive with sample antibodies that bind MEFA 12, are added. In Figure 8, the antigen reactive with the MEFA 12/sample antibody complex is a fusion between an SOD molecule and c22ks Δ47-L44W. The c22ks antigen is from the core region and includes amino acids Lys_{10} to Ser_{99} of the polyprotein, as well as a deletion of Arg47 normally present and a substitution of Leu for Trp at position 44. The antibody detection conjugate is the second HRP-labeled monoclonal anti-SOD antibody, described above.

The above-described antigen/antibody combination assays are particularly advantageous as both the HCV core antigen and antibodies to NS3/4a and/or core may be detected by the same support in the same assay. Moreover, as described above, additional HCV epitopes, such as SOD-fused to c100, 5-1-1, NS5 antigens, as well as a protein resulting from a frameshift in the core region of the polyprotein, such as

described in International Publication No. WO 99/63941, may be used in the combination cocktail to cover other non-structural epitopes of HCV.

In order to further an understanding of the invention, a more detailed discussion is provided below regarding production of antibodies for use in the subject immunoassays; production of polypeptides for use in the immunoassays; and methods of conducting the immunoassays.

Production of Antibodies for use in the HCV Immunoassays

As explained above, the assay utilizes various antibodies which are bound to a solid support (e.g., one or more anti-core antibodies), and that detect antigen/antibody complexes formed when HCV infection is present in the sample. These antibodies may be polyclonal or monoclonal antibody preparations, monospecific antisera, human antibodies, or may be hybrid or chimeric antibodies, such as humanized antibodies, altered antibodies, $F(ab')_2$ fragments, F(ab) fragments, Fv fragments, single-domain antibodies, dimeric or trimeric antibody fragment constructs, minibodies, or functional fragments thereof which bind to the antigen in question.

Antibodies are produced using techniques well known to those of skill in the art and disclosed in, for example, U.S. Patent Nos. 4,011,308; 4,722,890; 4,016,043; 3,876,504; 3,770,380; and 4,372,745. For example, polyclonal antibodies are generated by immunizing a suitable animal, such as a mouse, rat, rabbit, sheep or goat, with an antigen of interest. In order to enhance immunogenicity, the antigen can be linked to a carrier prior to immunization. Such carriers are well known to those of ordinary skill in the art. Immunization is generally performed by mixing or emulsifying the antigen in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). The animal is generally boosted 2-6 weeks later with one or more injections of the antigen in saline, preferably using Freund's incomplete adjuvant. Antibodies may also be generated by *in vitro* immunization, using methods known in the art. Polyclonal antiserum is then obtained from the immunized animal. See, e.g., Houghton et al., U.S. Patent No. 5,350,671, for a description of the production of anti-HCV polyclonal

antibodies.

Monoclonal antibodies are generally prepared using the method of Kohler and Milstein (1975) *Nature* 256:495-497, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the antigen. B-cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (*e.g.*, hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (e.g., as ascites in mice).

The production of various anti-HCV monoclonal antibodies has been described in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., International Publication No. WO 93/00365; commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818; and Kashiwakuma et al., U.S. Patent No. 5,871,904.

As explained above, antibody fragments which retain the ability to recognize the antigen of interest, will also find use in the subject immunoassays. A number of antibody fragments are known in the art which comprise antigen-binding sites capable of exhibiting immunological binding properties of an intact antibody molecule. For example, functional antibody fragments can be produced by cleaving a constant region, not responsible for antigen binding, from the antibody molecule, using e.g., pepsin, to produce $F(ab')_2$ fragments. These fragments will contain two antigen binding sites, but lack a portion of the constant region from each of the heavy chains. Similarly, if

desired, Fab fragments, comprising a single antigen binding site, can be produced, e.g., by digestion of polyclonal or monoclonal antibodies with papain. Functional fragments, including only the variable regions of the heavy and light chains, can also be produced, using standard techniques such as recombinant production or preferential proteolytic cleavage of immunoglobulin molecules. These fragments are known as F_v. See, e.g., Inbar et al. (1972) *Proc. Nat. Acad. Sci. USA* 69:2659-2662; Hochman et al. (1976) *Biochem* 15:2706-2710; and Ehrlich et al. (1980) *Biochem* 19:4091-4096.

A single-chain Fv ("sFv" or "scFv") polypeptide is a covalently linked V_H-V_L heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85:5879-5883. A number of methods have been described to discern and develop chemical structures (linkers) for converting the naturally aggregated, but chemically separated, light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. The sFv molecules may be produced using methods described in the art. See, e.g., Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85:5879-5883; U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Design criteria include determining the appropriate length to span the distance between the C-terminus of one chain and the N-terminus of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Suitable linkers generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility.

"Mini-antibodies" or "minibodies" will also find use with the present invention. Minibodies are sFv polypeptide chains which include oligomerization domains at their C-termini, separated from the sFv by a hinge region. Pack et al. (1992) *Biochem* 31:1579-1584. The oligomerization domain comprises self-associating α-helices, e.g., leucine zippers, that can be further stabilized by additional disulfide bonds. The

oligomerization domain is designed to be compatible with vectorial folding across a membrane, a process thought to facilitate *in vivo* folding of the polypeptide into a functional binding protein. Generally, minibodies are produced using recombinant methods well known in the art. See, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126.

Production of Antigens for use in the HCV Immunoassays

As explained above, the molecules of the present invention are generally produced recombinantly. Thus, polynucleotides encoding HCV antigens for use with the present invention can be made using standard techniques of molecular biology. For example, polynucleotide sequences coding for the above-described molecules can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing the gene, or by deriving the gene from a vector known to include the same. Furthermore, the desired gene can be isolated directly from viral nucleic acid molecules, using techniques described in the art, such as in Houghton et al., U.S. Patent No. 5,350,671. The gene of interest can also be produced synthetically, rather than cloned. The molecules can be designed with appropriate codons for the particular sequence. The complete sequence is then assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; and Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Thus, particular nucleotide sequences can be obtained from vectors harboring the desired sequences or synthesized completely or in part using various oligonucleotide synthesis techniques known in the art, such as site-directed mutagenesis and polymerase chain reaction (PCR) techniques where appropriate. See, e.g., Sambrook, *supra*. In particular, one method of obtaining nucleotide sequences encoding the desired sequences is by annealing complementary sets of overlapping synthetic oligonucleotides produced in a conventional, automated polynucleotide synthesizer, followed by ligation with an appropriate DNA ligase and amplification of the ligated nucleotide sequence via PVR. See, e.g., Jayaraman et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:4084-4088.

Additionally, oligonucleotide directed synthesis (Jones et al. (1986) *Nature* 54:75-82), oligonucleotide directed mutagenesis of pre-existing nucleotide regions (Riechmann et al. (1988) *Nature* 332:323-327 and Verhoeyen et al. (1988) *Science* 239:1534-1536), and enzymatic filling-in of gapped oligonucleotides using T_4 DNA polymerase (Queen et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:10029-10033) can be used under the invention to provide molecules having altered or enhanced antigen-binding capabilities, and/or reduced immunogenicity.

Once coding sequences have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Suitable vectors include, but are not limited to, plasmids, phages, transposons, cosmids, chromosomes or viruses which are capable of replication when associated with the proper control elements.

The coding sequence is then placed under the control of suitable control elements, depending on the system to be used for expression. Thus, the coding sequence can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence of interest is transcribed into RNA by a suitable transformant. The coding sequence may or may not contain a signal peptide or leader sequence which can later be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector. For example, enhancer elements may be used herein to increase expression levels of the constructs. Examples include the SV40 early gene enhancer (Dijkema et al. (1985) *EMBO J.* 4:761), the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma

Virus (Gorman et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:6777) and elements derived from human CMV (Boshart et al. (1985) *Cell* 41:521), such as elements included in the CMV intron A sequence (U.S. Patent No. 5,688,688). The expression cassette may further include an origin of replication for autonomous replication in a suitable host cell, one or more selectable markers, one or more restriction sites, a potential for high copy number and a strong promoter.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the molecule of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences in the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

As explained above, it may also be desirable to produce mutants or analogs of the antigen of interest. This is particularly true with NS3/4a. Methods for doing so are described in, e.g., Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276. Mutants or analogs of this and other HCV proteins for use in the subject assays may be prepared by the deletion of a portion of the sequence encoding the polypeptide of interest, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, and the like, are well known to those skilled in the art. See, e.g., Sambrook et al., supra; Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. (1987) *BioTechniques* 5:786; Zoller and Smith (1983) *Methods Enzymol.* 100:468; Dalbie-McFarland et al. (1982) *Proc. Natl. Acad. Sci USA* 79:6409.

The molecules can be expressed in a wide variety of systems, including insect, mammalian, bacterial, viral and yeast expression systems, all well known in the art.

For example, insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). Similarly, bacterial and mammalian cell expression systems are well known in the art and described in, e.g., Sambrook et al., *supra*. Yeast expression systems are also known in the art and described in, e.g., *Yeast Genetic Engineering* (Barr et al., eds., 1989) Butterworths, London.

A number of appropriate host cells for use with the above systems are also known. For example, mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human embryonic kidney cells, human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Nucleic acid molecules comprising nucleotide sequences of interest can be stably integrated into a host cell genome or maintained on a stable episomal element in a suitable host cell using various gene delivery techniques well known in the art. *See, e.g.*, U.S. Patent No. 5,399,346.

Depending on the expression system and host selected, the molecules are

produced by growing host cells transformed by an expression vector described above under conditions whereby the protein is expressed. The expressed protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the product can be purified directly from the media. If it is not secreted, it can be isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The recombinant production of various HCV antigens has been described. See, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778.

Immunodiagnostic Assays

Once produced, the above anti-core antibodies and NS3/4a antigens are placed on an appropriate solid support for use in the subject immunoassays. A solid support, for the purposes of this invention, can be any material that is an insoluble matrix and can have a rigid or semi-rigid surface. Exemplary solid supports include, but are not limited to, substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. Particular supports include plates, pellets, disks, capillaries, hollow fibers, needles, pins, solid fibers, cellulose beads, pore-glass beads, silica gels, polystyrene beads optionally cross-linked with divinylbenzene, grafted co-poly beads, polyacrylamide beads, latex beads, dimethylacrylamide beads optionally crosslinked with N-N'-bis-acryloylethylenediamine, and glass particles coated with a hydrophobic polymer.

If desired, the molecules to be added to the solid support can readily be functionalized to create styrene or acrylate moieties, thus enabling the incorporation of the molecules into polystyrene, polyacrylate or other polymers such as polyimide, polyacrylamide, polyethylene, polyvinyl, polydiacetylene, polyphenylene-vinylene, polypeptide, polysaccharide, polysulfone, polypyrrole, polyimidazole, polythiophene,

polyether, epoxies, silica glass, silica gel, siloxane, polyphosphate, hydrogel, agarose, cellulose, and the like.

In one context, a solid support is first reacted with the HCV anti-core antibodies and NS3/4a epitope (collectively called "the solid-phase components" herein), and optionally, one or more MEFAs, under suitable binding conditions such that the molecules are sufficiently immobilized to the support. Sometimes, immobilization to the support can be enhanced by first coupling the antigen and/or antibody to a protein with better solid phase-binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other reagents that can be used to bind molecules to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. (1992) *Bioconjugate Chem.* 3:2-13; Hashida et al. (1984) *J. Appl. Biochem.* 6:56-63; and Anjaneyulu and Staros (1987) *International J. of Peptide and Protein Res.* 30:117-124.

After reacting the solid support with the solid-phase components, any nonimmobilized solid-phase components are removed from the support by washing, and the support-bound components are then contacted with a biological sample suspected of containing HCV antibodies and antigens (collectively called "ligand molecules" herein) under suitable binding conditions. After washing to remove any nonbound ligand molecules, a second anti-core antibody, directed against a different epitope than the anti-core antibody bound to the support, is added under suitable binding conditions. The added anti-core antibody includes a detectable label, as described above, and acts to bind any core antigen that might be present in the sample which has reacted with the support-bound anti-core antibody. Also added are one or more antigens that can react with antibodies present in the sample that have, in turn, reacted with the NS3/4A epitope. As explained above, the antigen is typically derived from the NS3 region of the HCV polyprotein, and particularly from the c33c region of HCV. See, Houghton et al., U.S.

Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci.* (1989) 89:10011-10015; International Publication No. WO 93/00365; and commonly owned, allowed U.S. Patent Application Serial Nos. 08/403,590 and 08/444,818, for a description of this region and epitopes derived therefrom. A labeled antibody directed against this antigen is also added. The antibody will therefore bind the antigen, which has reacted with anti-NS3 antibodies present in the sample. For this purpose, the c33c epitope can be conveniently provided as a fusion between c33c and human superoxide dismutase (hSOD), produced recombinantly e.g., by methods described in Houghton et al., U.S. Patent No. 5,350,671. The nucleotide and amino acid sequences for human SOD are known and reported in Hallewell et al., U.S. Patent No. 5,710,033. A labeled antibody directed against human SOD can therefore be used to detect the presence of complexes formed between the NS3/4a epitope, any antibodies in the sample which react with this epitope, and HCV polypeptides which in turn bind the antibody in the sample.

If a MEFA is present on the solid support, one or more additional antigens, reactive with antibodies from the biological sample which are bound to antigens present on the MEFA, may also be added to the assay. Particularly useful in this context is an antigen derived from the core region of HCV, and more particularly, from the c22 antigen which includes 119 N-terminal core amino acids of the HCV polyprotein. One particular antigen derived from c22 is c22ks Δ47-L44W which includes amino acids Lys_{10} to Ser_{99} of the polyprotein, as well as a deletion of Arg47 normally present and a substitution of Leu for Trp at position 44. As with the c33c epitope described above, this antigen can be provided as a fusion with hSOD and the same labeled antibody, directed against human SOD, can be used to detect the presence of complexes formed between antibodies present in the sample and the NS3/4a epitope and/or the MEFA, which complexes are also bound with the HCV antigens (e.g., c33c and c22).

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with the solid-phase components. A biological sample containing or suspected of containing ligand molecules is then added to the coated wells. After a period of incubation sufficient to allow ligand-molecule binding to the immobilized solid-phase component, the plate(s) can be washed to remove unbound

moieties and a detectably labeled secondary binding molecule (labeled anti-core antibody), an NS3 epitope-containing molecule, and an antibody directed against the NS3 epitope-containing molecule added. These molecules are allowed to react with any captured sample antigen and antibody, the plate washed and the presence of the labeled antibodies detected using methods well known in the art.

The above-described assay reagents, including the immunoassay solid support with bound antibodies and antigens, as well as antibodies and antigens to be reacted with the captured sample, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

HCV Antigen/Antibody Combination Immunoassay

The present HCV antigen/antibody combination immunoassay was compared to other HCV assays to test the seroconversion detection limits and compare these limits to those obtained in other commercially available assays as follows.

A. Materials and Methods

Blood Samples: Panels of commercially available human blood samples were used. Such panels are available from, e.g., Boston Biomedica, Inc., West Bridgewater, MA (BBI); Bioclinical Partners, Franklin, MA (BCP); and North American Biologics, Inc., BocoRatan, FL (NABI). The days indicated in Tables 5 and 6 are days on which blood was collected from the subjects.

Monoclonal Antibodies: Monoclonal antibodies c11-3, c11-7 and c11-14 were obtained from Ortho Clinical Diagnostics, Raritan, New Jersey. The c11-3 and c11-7 antibodies are directed against an N-terminal portion of the core (amino acids 10-53, numbered relative to the HCV1 polyprotein). Monoclonal antibody c11-14 is directed against a C-terminal portion of the core (amino acids 120-130, numbered relative to the HCV1 polyprotein). The c11-14 antibody was conjugated to horse radish peroxidase (HRP) using standard procedures.

Monoclonal antibody 5A-3 is an anti-SOD antibody directed against amino acids 1 to 65 of SOD and was made using standard techniques. The antibody was conjugated to HRP as described above.

B. Antigens:

The c33c antigen (266 amino acids, amino acids 1192 to 1457 of the HCV1 polyprotein) was expressed as an internal SOD fusion polypeptide in *E. coli* by methods described for the synthesis of the 5-1-1 antigen (Choo, et al., *Science* (1989) 244:359-362). The recombinant antigen was purified as described in Chien, et al., *Proc. Natl. Acad. Sci.* (1989) 89:10011-10015. See, also, Houghton et al., U.S. Patent No. 5,350,671, for production protocols for SOD-c33c.

The NS3/4a epitope used in the assay is a conformational epitope having the sequence specified in Figure 3.

C. Immunoassay Formats:

The Abbott PRISM assay (Abbott Laboratories, Abbott Park, IL), is commercially available and is an antibody-based detection assay. The assay was performed using the manufacturer's instructions.

The ORTHO HCV Version 3.0 ELISA Test System (termed Ortho 3.0 assay herein, Ortho Clinical Diagnostics, Raritan, New Jersey) is an antibody-based detection assay. The assay was conducted using the manufacturer's instructions.

The Roche Amplicor assay (Roche, Pleasant, CA) is a commercially available PCR-based assay. The assay was performed using the manufacturer's instructions.

The Gen-Probe TMA assay (San Diego, CA) is a commercially available transcription-mediated amplification assay. The assay was performed using the manufacturer's instructions.

The Ortho antigen assay (Ortho Clinical Diagnostics, Raritan, New Jersey) is an antigen-based detection assay. The assay was performed using the manufacturer's instructions.

The subject HCV antigen/antibody combination immunoassay was performed as follows. 4mg/mL each of purified monoclonal antibodies C11-7 and C11-3 in 1x phosphate-buffered saline (PBS), pH 7.4 were combined and mixed well. 90ng of the NS3/4a recombinant antigen was added to the same coating buffer. The solution was mixed for 30 minutes prior to coating. 200mL of the above solution was added per well to 96-well Costar medium binding microtiter plates (Corning, Inc.) Plates were incubated at 15-30°C for 16-24 hours. Plates were washed two times with dH_20, followed with 300µL/well postcoat buffer (1% bovine serum albumin (BSA), 1x PBS) for 1 hour and 300µl/well stability buffer (1x PBS, 1% BSA, mannitol, polyethylene glycol (PEG), gelatin) for 1 hour. Plates were aspirated and dried at 4°C in a lyophilizer for 24 hours. Plates were pouched with desiccant.

To conduct the antigen/antibody combination immunoassay, 100µL of enhanced lysis buffer (1% N-laurylsarcosine, 0.65M NaCl, 50mg/mL mouse IgG technical grade (Sigma, St. Louis, MO), 1% BSA sulfhydryl-modified (Bayer), 0.1% Casein) were added to the plate. 100mL of sample were then added. This was incubated on a shaker at 40°C for one hour. The plates were washed six times with 1x PBS, 0.1% Tween-20,

on an Ortho Plate Washer. 200mL conjugate solution (1:75 dilution c11-14-HRP with 250ng/assay SOD-c33c antigen plus 1:5000 dilution mouse anti-SOD-HRP in HCV 3.0 sample diluent (from ORTHO HCV Version 3.0 ELISA Test System, Ortho Clinical Diagnostics, Raritan, New Jersey) without SOD extract, all prepared 30 minutes prior to addition). The solution was incubated 45 minutes with shaking at 40°C. This was washed six times, as above, and 200mL substrate solution (1 OPD tablet/10mL) was added. The OPD tablet contains *o*-phenylenediamine dihydrochloride and hydrogen peroxide for horse radish peroxidase reaction color development and is available from Sigma, St. Louis, MO. This was incubated 30 minutes at 15-30°C in the dark. The reaction was stopped by addition of 50mL 4N H_2SO_4 and the plates were read at 492nm, relative to absorbance at 690nm as control.

D. Results:

The results of the various assays are shown in Tables 5 and 6, which depict two separate experiments done on blood samples exposed to HCV infection as indicated. Shaded areas indicate detection of virus. As shown in below, Chiron's combination antigen/antibody assay detected seroconversion in all samples, while all other antibody- and antigen-based assays failed to detect seroconversion in at least one sample. In particular, neither of the antibody-based assays detected seroconversion until at least day 18 (Table 5). Table 6 shows that neither of the antibody-based assays detected the presence of HCV infection at day 22. Moreover, the Ortho antigen-based assay failed to detect seroconversion from days 85 on.

Thus, based on the above results, it is clear that the novel combination antibody/antigen assay reduces the number of false negatives obtained using other conventional antibody- and antigen-based assays.

Table 5
HCV Seroconversion

Days	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	$>5 \times 10^5$	9.25	18.6	2.8
4	0.1	0.0	$>5 \times 10^5$	9.29	19.0	3.1
7	0.1	0.0	$>5 \times 10^5$	9.52	22.3	1.5
13	0.3	0.1	$>5 \times 10^5$	9.59	26.2	1.7
18	1.3	0.4	$>5 \times 10^5$	9.70	15.9	1.2
21	2.2	1.0	$>5 \times 10^5$	9.39	11.3	1.5
164	4.2	4.4	4×10^4	9.28	0.11	2.5

Table 6
HCV Seroconversion

Days	Abbott PRISM	Ortho 3.0	Roche Amplicor	Gen-Probe TMA	Ortho Ag	Chiron Ag/Ab
0	0.1	0.0	BLD		0.11	0.5
13	0.1	0.0	$>5 \times 10^5$		44.0	3.0
20	0.1	0.0	$>5 \times 10^5$		24.2	1.3
22	0.3	0.0	$>5 \times 10^5$		29.2	1.6
85	5.4	4.7	BQR		0.06	1.1
131	4.3	4.7	BQR		0.09	1.0
135	4.6	4.7	3×10^3		0.09	1.2
138	5.5	4.7	BLD		0.08	1.2
146	5.9	4.7	BLD		0.11	2.1
152	5.2	4.7	BQR		0.07	1.8

EXAMPLE 2

Production of an NS3/4a Conformational Epitope with

Thr to Pro and Ser to Ile Substitutions

A conformational epitope of NS3/4a was obtained as follows. This epitope has the sequence specified in Figures 4A through 4D and differs from the native sequence at positions 403 (amino acid 1428 of the HCV-1 full-length sequence) and 404 (amino acid 1429 of the HCV-1 full-length sequence). Specifically, the Thr normally occurring at position 1428 of the native sequence has been mutated to Pro and Ser which occurs at position 1429 of the native sequence has been mutated to Ile.

In particular, the yeast expression vector used was pBS24.1, described above. Plasmid pd.hcv1a.ns3ns4aPI, which encoded a representative NS3/4a epitope used in the subject immunoassays, was produced as follows. A two step procedure was used. First, the following DNA pieces were ligated together: (a) synthetic oligonucleotides which

would provide a 5' *Hind*III cloning site, followed by the sequence ACAAAACAAA, the initiator ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*I site at amino acid 1046; (b) a 683 bp *Bgl*I-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI; and (c) a pSP72 vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65332) which had been digested with *Hind*III and *Cla*I, dephosphorylated, and gel-purified. Plasmid pAcHLTns3ns4aPI was derived from pAcHLT, a baculovirus expression vector commercially available from BD Pharmingen (San Diego, CA). In particular, a pAcHLT *Eco*RI-*Pst*I vector was prepared, as well as the following fragments: *Eco*RI- *Alw*nI, 935 bp, corresponding to amino acids 1027-1336 of the HCV-1 genome; *Alw*nI-*Sac*II, 247 bp, corresponding to amino acids 1336-1419 of the HCV-1 genome; *Hinf*I-*Bgl*I, 175 bp, corresponding to amino acids 1449-1509 of the HCV-1 genome; *Bgl*I-*Pst*I, 619 bp, corresponding to amino acids 1510-1711 of the HCV-1 genome, plus the transcription termination codon. A *Sac*II -*Hinf*I synthetically generated fragment of 91 bp, corresponding to amino acids 1420-1448 of the HCV-1 genome and containing the PI mutations (Thr-1428 mutated to Pro, Ser-1429 mutated to Ile), was ligated with the 175 bp *Hinf*I-*Bgl*I fragment and the 619 bp *Bgl*I-*Pst*I fragment described above and subcloned into a pGEM-5Zf(+) vector digested with *Sac*II and *Pst*I. pGEM-5Zf(+) is a commercially available *E. coli* vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65308). After transformation of competent HB101 cells, miniscreen analysis of individual clones and sequence verification, an 885 bp *Sac*II-*Pst*I fragment from pGEM5.PI clone2 was gel-purified. This fragment was ligated with the *Eco*RI-*Alw*nI 935 bp fragment, the *Alw*nI-*Sac*II 247 bp fragment and the pAcHLT *Eco*RI-*Pst*I vector, described above. The resultant construct was named pAcHLTns3ns4aPI.

The ligation mixture above was transformed into HB101-competent cells and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual clones led to the identification of putative positives, two of which were amplified. The plasmid DNA for pSP72 1aHC, clones #1 and #2 were prepared with a Qiagen Maxiprep kit and were sequenced.

Next, the following fragments were ligated together: (a) a 761 bp *Hind*III-*Cla*I

fragment from pSP721aHC #1 (pSP72.1aHC was generated by ligating together the following: pSP72 which had been digested with *Hind*III and *Cla*I, synthetic oligonucleotides which would provide a 5' *Hind*III cloning site, followed by the sequence ACAAAACAAA, the initiation codon ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*II site at amino acid 1046, and a 683 bp *Bgl*II-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI); (b) a 1353 bp *Bam*HI-*Hind*III fragment for the yeast hybrid promoter ADH2/GAPDH; (c) a 1320 bp *Cla*I-*Sal*I fragment (encoding HCV1a amino acids 1046-1711 with Thr 1428 mutated to Pro and Ser 1429 mutated to Ile) from pAcHLTns3ns4aPI; and (d) the pBS24.1 yeast expression vector which had been digested with *Bam*HI and *Sal*I, dephosphorylated and gel-purified. The ligation mixture was transformed into competent HB101 and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual colonies led to the identification of clones with the expected 3446 bp *Bam*HI-*Sal*I insert which was comprised of the ADH2/GAPDH promoter, the initiator codon ATG and HCV1a NS3/4a from amino acids 1027-1711 (shown as amino acids 1-686 of Figures 4A-4D), with Thr 1428 (amino acid position 403 of Figures 4A-4D) mutated to Pro and Ser 1429 (amino acid position 404 of Figures 4A-4D) mutated to Ile. The construct was named pd.HCV1a.ns3ns4aPI (see, Figure 5).

S. cerevisiae strain AD3 was transformed with pd.HCV1a.ns3ns4aPI and single transformants were checked for expression after depletion of glucose in the medium. The recombinant protein was expressed at high levels in yeast, as detected by Coomassie blue staining and confirmed by immunoblot analysis using a polyclonal antibody to the helicase domain of NS3.

EXAMPLE 3

Purification of NS3/4a Conformational Epitope

The NS3/4a conformational epitope was purified as follows. *S. cerevisiae* cells from above, expressing the NS3/4a epitope were harvested as described above. The cells were suspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin) and lysed in a Dyno-Mill (Wab Willy A. Bachofon, Basel, Switzerland) or equivalent apparatus using glass beads, at a ratio of 1:1:1 cells:buffer:0.5 mm glass beads. The lysate was centrifuged at 30100 x g for 30 min at 4°C and the pellet containing the insoluble protein fraction was added to wash buffer (6 ml/g start cell pellet weight) and rocked at room temperature for 15 min. The wash buffer consisted of 50 mM $NaPO_4$ pH 8.0, 0. 3 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 0.05% octyl glucoside, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. Cell debris was removed by centrifugation at 30100 x g for 30 min at 4°C. The supernatant was discarded and the pellet retained.

Protein was extracted from the pellet as follows. 6 ml/g extraction buffer was added and rocked at room temperature for 15 min. The extraction buffer consisted of 50 mM Tris pH 8.0, 1 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. This was centrifuged at 30100 x g for 30 min at 4°C. The supernatant was retained and ammonium sulfate added to 17.5% using the following formula: volume of supernatant (ml) multiplied by x% ammonium sulfate/(1 - x% ammonium sulfate) = ml of 4.1 M saturated ammonium sulfate to add to the supernatant. The ammonium sulfate was added dropwise while stirring on ice and the solution stirred on ice for 10 min. The solution was centrifuged at 17700 x g for 30 min at 4°C and the pellet retained and stored at 2°C to 8°C for up to 48 hrs.

The pellet was resuspended and run on a Poly U column (Poly U Sepharose 4B, Amersham Pharmacia) at 4°C as follows. Pellet was resuspended in 6 ml Poly U equilibration buffer per gram of pellet weight. The equilibration buffer consisted of 25 mM HEPES pH 8.0, 200 mM NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. The solution was rocked at 4°C for 15 min and centrifuged at 31000 x g for 30 min at 4°C.

A Poly U column (1 ml resin per gram start pellet weight) was prepared. Linear flow rate was 60 cm/hr and packing flow rate was 133% of 60 cm/hr. The column was equilibrated with equilibration buffer and the supernatant of the resuspended ammonium sulfate pellet was loaded onto the equilibrated column. The column was washed to baseline with the equilibration buffer and protein eluted with a step elution in the following Poly U elution buffer: 25 mM HEPES pH 8.0, 1 M NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. Column eluate was run on SDS-PAGE (Coomassie stained) and aliquots frozen and stored at -80°C. The presence of the NS3/4a epitope was confirmed by Western blot, using a polyclonal antibody directed against the NS3 protease domain and a monoclonal antibody against the 5-1-1 epitope (HCV 4a).

Additionally, protease enzyme activity was monitored during purification as follows. An NS4A peptide (KKGSVVIVGRIVLSGKPAIIPKK), and the sample containing the NS3/4a conformational epitope, were diluted in 90 µl of reaction buffer (25 mM Tris, pH 7.5, 0.15M NaCl, 0.5 mM EDTA, 10% glycerol, 0.05 n-Dodecyl B-D-Maltoside, 5 mM DTT) and allowed to mix for 30 minutes at room temperature. 90 µl of the mixture were added to a microtiter plate (Costar, Inc., Corning, NY) and 10 µl of HCV substrate (AnaSpec, Inc., San Jose CA) was added. The plate was mixed and read on a Fluostar plate reader. Results were expressed as relative fluorescence units (RFU) per minute.

Using these methods, the product of the 1 M NaCl extraction contained 3.7 RFU/min activity, the ammonium sulfate precipitate had an activity of 7.5 RFU/min and the product of the Poly U purification had an activity of 18.5 RFU/min.

EXAMPLE 4

Competition Studies

The following competition study was conducted in order to assess whether the NS3/4a conformational epitope detected different antibodies than other HCV antigens. In particular, the NS3/4a antigen was compared with the c200 antigen as follows.

0.5 µg and 1.0 µg of NS3/4a, produced as described above, or c200 (*Hepatology*

(1992) 15:19-25, available in the ORTHO HCV Version 3.0 ELISA Test System, Ortho-Clinical Diagnostics, Raritan, New Jersey), were mixed with 20 µl of sample PHV914-5 (an early seroconversion bleed obtained from blood of an infected individual) in a total volume of 220 µl (1 x PBS). The mixture was incubated for 1 hour in microwells at 37°C. The mixture was then transferred to NS3/4a-coated plates and incubated for 1 hour at 37°C. Plates were washed and assayed as follows.

1 µg of c200 antigen was added to 10 µl of sample PHV914-5 in a total volume of about 220 µl. The mixture was incubated for 1 hour in a micro well at 37°C and 200 µl transferred to an NS3/4a-coated plate (100 ng/assay) and incubated for 1 hour at 37°C. Plates were washed five times with 1 x PBS, 0.1% Tween-20. 200 µl of conjugate solution (described above) were added, and the plates incubated and assayed. Controls which consisted of PHV914-5 and 1 x PBS (without antigen) were also treated as above.

Results are shown in Table 7. Percent inhibition results shown in column 4 are calculated as column 3 minus (column 2 divided by column 3 times 100). As can be seen, the data show that NS34a is neutralized by early seroconversion antibodies and c200 is not. A strong signal was achieved when antibodies in PHV914-5 c33c early seroconversion panel member reacted with the NS34a coated on the plate. The c200 antigen was not neutralized by these antibodies. This is shown in the top panel of Table 7. When NS34a was mixed with the PHV914-5 sample, it was neutralized and therefore no antibodies were present in the sample to react with NS34a that was coated on the microplate. The data indicate that NS34a may be detecting a different class of antibodies than is detected by c200.

Competition Studies to Show NS34a Antigen Detects Different Antibodies in Early c33c Seroconversion Panel Compared to c200 Antigen

	1	2	3	4
			Control 1X PBS	% Inhibition
c200	+	PHV914-5 s	s	
1ug		1.450	1.645	12
1ug		1.545	1.687	8
0.5ug		1.557	1.913	19
0.5ug		1.719	1.804	5
NS3/4a	+	PHV914-5 s	s	
1ug		0.054	1.599	97
1ug		0.037	1.677	98
0.5ug		0.066	1.672	96
0.5ug		NA	1.524	NA

TABLE 7

EXAMPLE 5

Stability Studies of NS3/4a Conformational Epitope

To assess the role of stability of the NS3/4a epitope to assay performance, the following study was done to determine NS3/4a immunoreactivity versus time at room temperature. Small aliquots of stock NS3/4a were allowed to sit at room temperature and then frozen at intervals as shown in Table 8. All vials were coated simultaneously and tested against two early NS3 seroconversion panels.

As can be seen in Table 8, the NS3/4a stock is not stable and immunoreactivity decreases with time. In addition, maintaining NS3/4a conformation is necessary for immunoreactivity.

Further stability studies were conducted as follows. Two conformational monoclonal antibodies made against NS3/4a using standard procedures were substituted for anti-HCV early seroconversion panels. Stock NS3/4a vials were stored at room temperature at time intervals 3, 6 and 24 hours. The NS3/4a from the frozen vials was coated at 90 ng/ml and assayed using the procedure described above. Results suggested that the two monoclonals were indeed conformational and their reactivity was sensitive to the handling of stock NS3/4a antigen at room temperature. The reactivity of a positive control monoclonal antibody did not change.

Time (hrs)	0	6	21.4	29	35.5	46	52	control
	A	D	G	H	I	K	N	Reference
	s/co	s/co	s/co	s/co	s/co	s/co	s/co	s/co
PHV 904-1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PHV 904-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PHV 904-3	[illegible]	0.3	0.1	0.0	0.0	0.0	0.0	[illegible]
PHV 904-4	[illegible]	[illegible]	0.2	0.1	0.1	0.1	0.1	[illegible]
PHV 904-5	[illegible]	[illegible]	0.7	0.6	0.3	0.2	0.3	[illegible]
PHV 904-6	[illegible]	[illegible]	[illegible]	[illegible]	0.6	0.5	0.6	[illegible]
PHV 904-7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.5	0.7	[illegible]
PHV 914-1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PHV 914-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PHV 914-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PHV 914-4	0.5	0.1	0.0	0.0	0.0	0.0	0.0	0.7
PHV 914-5	[illegible]	0.4	0.0	0.0	0.0	0.0	0.0	[illegible]
PHV 914-6	[illegible]	0.4	0.0	0.0	0.0	0.0	0.0	[illegible]
PHV 914-7	[illegible]	0.5	0.1	0.1	0.0	0.0	0.0	[illegible]
PHV 914-8	[illegible]	0.7	0.1	0.1	0.1	0.1	0.1	[illegible]
Enzyme RFU/min	8.75	4.14	3.08	1.88	1.75	1.75	0.75	

TABLE 8

EXAMPLE 6

Immunoreactivity of NS3/4a Conformational Epitope Verus Denatured NS3/4a

The immunoreactivity of the NS3/4a conformational epitope, produced as described above, was compared to NS3/4a which had been denatured by adding SDS to the NS3/4a conformational epitope preparation to a final concentration of 2%. The denatured NS3/4a and conformational NS3/4a were coated onto microtiter plates as described above. The c200 antigen (*Hepatology* (1992) 15:19-25, available in the ORTHO HCV Version 3.0 ELISA Test System, Ortho-Clinical Diagnostics, Raritan, New Jersey) was also coated onto microtiter plates. The c200 antigen was used as a comparison it is presumed to be non-conformational due to the presence of reducing agent (DTT) and detergent (SDS) in its formulation.

The immunoreactivity was tested against two early HCV seroconversion panels, PHV 904 and PHV 914 (commercially available human blood samples from Boston Biomedica, Inc., West Bridgewater, MA). The results are shown in Table 9. The data suggest that the denatured or linearized form of NS3/4a (as well as c200) does not detect early seroconversion panels as early as the NS3/4a conformational epitope.

	NS3/4a vs. denatured NS3/4a *Spiked 2% SDS to stock NS3/4a							
		NS3/4a	dNS3/4a*	c200		NS3/4a	dNS3/4a*	c200
		OD	OD	OD		s/co	s/co	s/co
HCV	PHV 904-1	0.012	0.012	0.009		0.02	0.02	0.01
Seroconversions	PHV 904-2	0.011	0.009	0.008		0.02	0.01	0.01
	PHV 904-3	1.124	0.071	0.045		1.30	0.11	0.07
	PHV 904-4	2.401	0.273	0.129		2.85	0.44	0.21
	PHV 904-5	3.022	0.793	0.347		4.85	1.28	0.57
	PHV 904-6	2.711	1.472	0.774		4.09	2.37	1.30
	PHV 904-7	3.294	1.860	0.943		5.21	2.99	1.65
	PHV 914-1	0.006	0.004	0.001		0.01	0.01	0.00
	PHV 914-2	0.005	0.004	0.002		0.01	0.01	0.00
	PHV 914-3	0.098	0.003	0.001		0.16	0.00	0.00
	PHV 914-4	1.118	0.006	0.004		1.79	0.01	0.01
	PHV 914-5	2.035	0.044	0.022		3.26	0.07	0.04
	PHV 914-6	2.092	0.074	0.025		3.35	0.12	0.04
	PHV 914-7	2.519	0.281	0.132		4.01	0.45	0.22
	PHV 914-8	2.746	0.907	0.500		4.40	1.45	0.82
	PHV 914-9	3.084	1.730	0.931		4.94	2.76	1.53
HCV 3.0	Neg.Cont.	0.023	0.024	0.008				
Controls	Neg.Cont.	0.027	0.024	0.007				
	Neg.Cont.	0.021	0.017	0.005				
	average	0.024	0.022	0.007				
	cutoff	0.624	0.622	0.607				
	Pos. Cont.	1.239	0.903	0.575		1.99	1.45	0.95
	Pos. Cont.	1.445	0.916	0.614		2.32	1.47	1.01

TABLE 9

Immunoreactivity of the conformational epitope was also tested using monoclonal antibodies to NS3/4a, made using standard procedures. These monoclonal antibodies were then tested in the ELISA format against NS3/4a and denatured NS3/4a and c200 antigen. The data show that anti-NS3/4a monoclonals react to the NS3/4a and denatured NS3/4a in a similar manner to the seroconversion panels shown in Table 10. This result also provides further evidence that the NS3/4a is conformational in nature as monoclonal antibodies can be made which are similar in reactivity to the early c33c seroconversion panels .

Table 10

		Plate		
		NS3/4a	dNS3/4a	c200
Monoclonal		OD	OD	OD
4B9/E3	1:100	1.820	0.616	0.369
	1:1000	1.397	0.380	0.246
	1:10000	0.864	0.173	0.070
	1:20000	0.607	0.116	0.085
5B7/D7	1:100	2.885	0.898	0.436
	1:1000	2.866	0.541	0.267
	1:10000	1.672	0.215	0.086
	1:20000	1.053	0.124	0.059
1A8/H2	1:100	1.020	0.169	0.080
	1:1000	0.921	0.101	0.043
	1:10000	0.653	0.037	0.013
	1:20000	0.337	0.027	0.011

Accordingly, novel HCV detection assays have been disclosed. From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the disclosure herein.

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Claims

1. An immunoassay solid support comprising at least one hepatitis C virus (HCV) anti-core antibody and at least one isolated HCV NS3/4a epitope bound thereto.

2. The immunoassay solid support of claim 1, comprising at least two HCV anti-core antibodies bound thereto.

3. The immunoassay solid support of claim 1, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen.

4. The immunoassay solid support of claim 3, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence.

5. The immunoassay solid support of claim 1, wherein said at least one anti-core antibody is a monoclonal antibody.

6. The immunoassay solid support of claim 1, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

7. The immunoassay solid support of claim 1, further comprising a multiple epitope fusion antigen bound thereto.

8. The immunoassay solid support of claim 7, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F.

9. An immunoassay solid support comprising two hepatitis C virus (HCV) anti-core monoclonal antibodies and an HCV NS3/4a conformational epitope comprising the

amino acid sequence depicted in Figures 4A-4D, bound thereto.

10. The immunoassay solid support of claim 9, wherein said two anti-core antibodies are directed against an N-terminal region of the HCV core antigen.

11. The immunoassay solid support of claim 10, wherein said two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence.

12. An immunoassay solid support comprising two hepatitis C virus (HCV) anti-core monoclonal antibodies, an HCV NS3/4a conformational epitope comprising the amino acid sequence depicted in Figures 4A-4D, and a multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, bound thereto.

13. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 1;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to said at least one anti-core antibody and said NS3/4a epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) an antigen that reacts with an HCV antibody from the biological sample reactive with said NS3/4a epitope; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with the antigen of (ii);

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

14. The method of claim 13, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

15. The method of claim 14, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

16. The method of claim 13, wherein said antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c33c region of the HCV polyprotein.

17. The method of claim 16, wherein the c33c epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

18. The method of claim 13, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

19. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 2;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies and said NS3/4a epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is

directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequence;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

20. The method of claim 19, wherein said NS3/4a epitope is a conformational epitope and comprises the amino acid sequence depicted in Figures 4A-4D.

21. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 9;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies and said NS3/4a conformational epitope, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequence;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

22. The method of claim 21, wherein said at least two anti-core antibodies are

directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

23. The method of claim 22, wherein said at least two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

24. A method of detecting hepatitis C virus (HCV) infection in a biological sample, said method comprising:

(a) providing an immunoassay solid support according to claim 7;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to said at least one anti-core antibody, said NS3/4a epitope, and said multiple epitope fusion antigen;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least one anti-core antibody bound to the solid support; (ii) first and second antigens that react with an HCV antibody from the biological sample reactive with said NS3/4a epitope and said multiple epitope fusion antigen, respectively; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with the antigens of (ii);

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

25. The method of claim 24, wherein said at least one anti-core antibody is directed against an N-terminal region of the HCV core antigen and said first detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core

antigen.

26. The method of claim 25, wherein said at least one anti-core antibody is directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

27. The method of claim 24, wherein said first antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c33c region of the HCV polyprotein.

28. The method of claim 27, wherein the c33c epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

29. The method of claim 24, wherein said second antigen that reacts with an HCV antibody from the biological sample comprises an epitope from the c22 region of the HCV polyprotein.

30. The method of claim 29, wherein the epitope from the c22 region comprises amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence, wherein said epitope is fused with a human superoxide dismutase (hSOD) amino acid sequence and the second detectably labeled antibody is reactive with said hSOD amino acid sequence.

31. The method of claim 24, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F.

32. A method of detecting hepatitis C virus (HCV) infection in a biological

sample, said method comprising:

(a) providing an immunoassay solid support according to claim 12;

(b) combining a biological sample with said solid support under conditions which allow HCV antigens and antibodies, when present in the biological sample, to bind to the said at least two anti-core antibodies, said NS3/4a conformational epitope, and said multiple epitope fusion antigen, respectively;

(c) adding to the solid support from step (b) under complex forming conditions (i) a first detectably labeled antibody, wherein said first detectably labeled antibody is a detectably labeled HCV anti-core antibody, wherein said labeled anti-core antibody is directed against a different HCV core epitope than the at least two anti-core antibodies bound to the solid support; (ii) an epitope from the c33c region of the HCV polyprotein fused to an hSOD amino acid sequence and an epitope from the c22 region of the HCV polyprotein fused to an hSOD amino acid sequence; and (iii) a second detectably labeled antibody, wherein said second detectably labeled antibody is reactive with said hSOD amino acid sequences;

(d) detecting complexes formed between the antibodies and antigens, if any, as an indication of HCV infection in the biological sample.

33. The method of claim 32, wherein said at least two anti-core antibodies are directed against an N-terminal region of the HCV core antigen and said detectably labeled HCV anti-core antibody is directed against a C-terminal region of the HCV core antigen.

34. The method of claim 33, wherein said at least two anti-core antibodies are directed against amino acids 10-53 of HCV, numbered relative to the HCV1 polyprotein sequence and said detectably labeled HCV anti-core antibody is directed against amino acids 120-130 of HCV, numbered relative to the HCV1 polyprotein sequence.

35. The method of claim 32, wherein the epitope from the c22 region comprises amino acids Lys_{10} to Ser_{99} of the HCV polyprotein, with a deletion of Arg47 and a

substitution of Leu for Trp at position 44, numbered relative to the HCV1 polyprotein sequence.

36. An immunodiagnostic test kit comprising the immunoassay solid support of any of claims 1-12, and instructions for conducting the immunodiagnostic test.

37. A method of producing an immunoassay solid support, comprising:
(a) providing a solid support; and
(b) binding at least one hepatitis C virus (HCV) anti-core antibody and at least one isolated HCV NS3/4a conformational epitope thereto.

38. A method of producing an immunoassay solid support, comprising:
(a) providing a solid support; and
(b) binding two hepatitis C virus (HCV) anti-core antibodies and an isolated HCV NS3/4a conformational epitope thereto.

39. The method of either of claims 38 or 39, further comprising binding at least one multiple epitope fusion antigen to the solid support.

40. A multiple epitope fusion antigen comprising the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 80% sequence identity thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

41. The multiple epitope fusion antigen of claim 40, wherein said multiple epitope fusion antigen comprises the amino acid sequence depicted in Figures 7A-7F, or an amino acid sequence with at least 90% sequence identity thereto which reacts specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

42. The multiple epitope fusion antigen of claim 40, wherein said multiple epitope fusion antigen consists of the amino acid sequence depicted in Figures 5A-5F.

43. A polynucleotide comprising a coding sequence for the multiple epitope fusion antigen of any of claims 40-42.

44. A recombinant vector comprising:

(a) a polynucleotide according to claim 43;

(b) and control elements operably linked to said polynucleotide whereby the coding sequence can be transcribed and translated in a host cell.

45. A host cell transformed with the recombinant vector of claim 44.

46. A method of producing a recombinant multiple epitope fusion antigen comprising:

(a) providing a population of host cells according to claim 45; and

(b) culturing said population of cells under conditions whereby the multiple epitope fusion antigen encoded by the coding sequence present in said recombinant vector is expressed.

HCV Genome and Recombinant Proteins

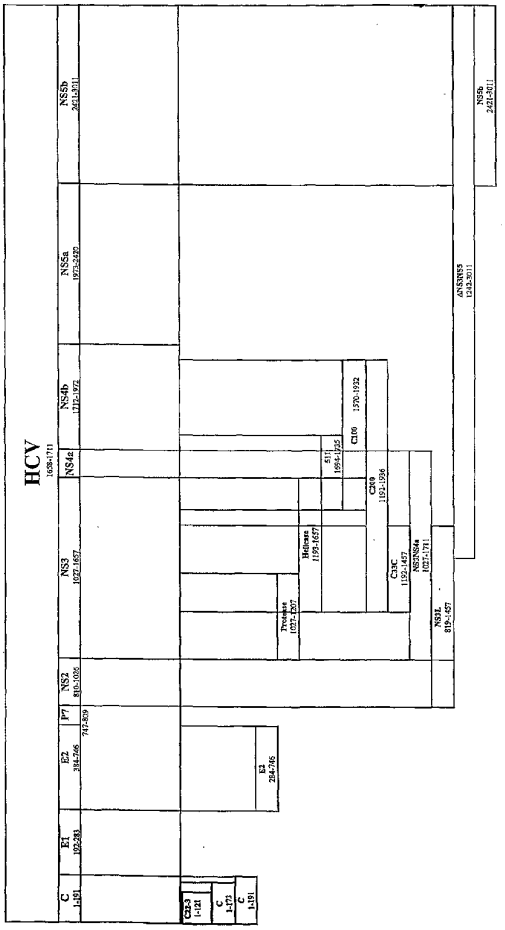


FIG. 1

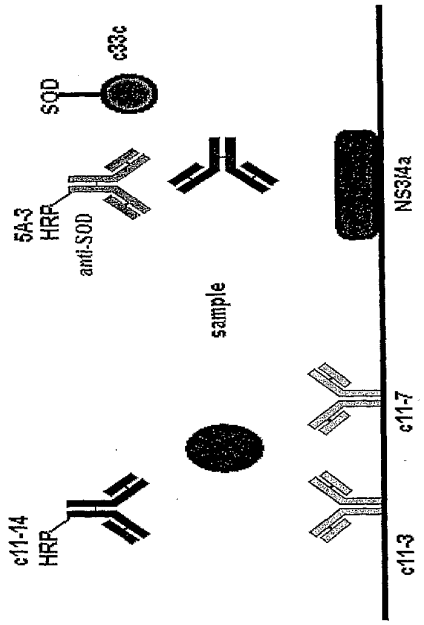


FIG. 2

```
MSPIDPMGHHHHHHGRRRASVAAGILVPRGSPGLDGICSIEEPAPITAYAQQTRGLLGCIITSLTGRDKNQVE  73
GEVQIVSTAAQTFLATCINGVCWTVYHGAGTRTIASPKGPVIQMYTNVDQDLVGWPASQGTRSLTPCTCGSSD 146
LYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSAGGPLLCPAGHAVGIFRAAVCTRGVAKAVDFIPVENLE 219
TTMRSPVFTDNSSPPVVPQSFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHGID 292
PNIRTGVRTITTGSPITYSTYGKFLADGGCSGGAYDIIICDECHSTDATSILGIGTVLDQAETAGARLVVLAT 365
ATPPGSVTVPHPNIEEVALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKLVALGINAVAYYRGL 438
DVSVIPTIGDVVVVATDALMTGYTGDFDSVIDCNTCVTQTVDFSLDPTFTIETITLPQDAVSRTQRRGRTGRG 511
KPGIYRFVAPGERPSGMFDSSVLCECYDAGCAWYELTPAETTVRLRAYMNTPGLPVCQDHLEFWEGVFTGLTH 584
IDAHFLSQTKQSGENLPYLVAYQATVCARAQAPPPSWDQMWKCLIRLKPTLHGPTPLLYRLGAVQNEITLTHP 657
VTKYIMTCMSADLEVVTSTWVLVGGVLAALAAYCLSTGCVVIVGRVVLSGKPAIIPDREVLYREFDEMEEC  728
```

FIG. 3

```
                    1                                                  10
                    M   A   P   I   T   A   Y   A   Q   Q
                   ATG GCG CCC ATC ACG GCG TAC GCC CAG CAG

                                       20
  T   R   G   L   L   G   C   I   I   T   S   L   T   G   R
 ACA AGG GGC CTC CTA GGG TGC ATA ATC ACC AGC CTA ACT GGC CGG

                   30                                                  40
  D   K   N   Q   V   E   G   E   V   Q   I   V   S   T   A
 GAC AAA AAC CAA GTG GAG GGT GAG GTC CAG ATT GTG TCA ACT GCT

                                       50
  A   Q   T   F   L   A   T   C   I   N   G   V   C   W   T
 GCC CAA ACC TTC CTG GCA ACG TGC ATC AAT GGG GTG TGC TGG ACT

                   60                                                  70
  V   Y   H   G   A   G   T   R   T   I   A   S   P   K   G
 GTC TAC CAC GGG GCC GGA ACG AGG ACC ATC GCG TCA CCC AAG GGT

                                       80
  P   V   I   Q   M   Y   T   N   V   D   Q   D   L   V   G
 CCT GTC ATC CAG ATG TAT ACC AAT GTA GAC CAA GAC CTT GTG GGC

                   90                                                  100
  W   P   A   P   Q   G   S   R   S   L   T   P   C   T   C
 TGG CCC GCT CCG CAA GGT AGC CGA TCA TTG ACA CCC TGC ACT TGC

                                       110
  G   S   S   D   L   Y   L   V   T   R   H   A   D   V   I
 GGC TCC TCG GAC CTT TAC CTG GTC ACG AGG CAC GCC GAT GTC ATT

                  120                                                  130
  P   V   R   R   R   G   D   S   R   G   S   L   L   S   P
 CCC GTG CGC CGG CGG GGT GAT AGC AGG GGC AGC CTG CTG TCG CCC

                                       140
  R   P   I   S   Y   L   K   G   S   S   G   G   P   L   L
 CGG CCC ATT TCC TAC TTG AAA GGC TCC TCG GGG GGT CCG CTG TTG

                  150                                                  160
  C   P   A   G   H   A   V   G   I   F   R   A   A   V   C
 TGC CCC GCG GGG CAC GCC GTG GGC ATA TTT AGG GCC GCG GTG TGC

                                       170
  T   R   G   V   A   K   A   V   D   F   I   P   V   E   N
 ACC CGT GGA GTG GCT AAG GCG GTG GAC TTT ATC CCT GTG GAG AAC

                  180                                                  190
  L   E   T   T   M   R   S   P   V   F   T   D   N   S   S
 CTA GAG ACA ACC ATG AGG TCC CCG GTG TTC ACG GAT AAC TCC TCT
```

FIG. 4A

```
                                                    200
 P   P   V   V   P   Q   S   F   Q   V   A   H   L   H   A
CCA CCA GTA GTG CCC CAG AGC TTC CAG GTG GCT CAC CTC CAT GCT

                    210                             220
 P   T   G   S   G   K   S   T   K   V   P   A   A   Y   A
CCC ACA GGC AGC GGC AAA AGC ACC AAG GTC CCG GCT GCA TAT GCA

                                                    230
 A   Q   G   Y   K   V   L   V   L   N   P   S   V   A   A
GCT CAG GGC TAT AAG GTG CTA GTA CTC AAC CCC TCT GTT GCT GCA

                    240                             250
 T   L   G   F   G   A   Y   M   S   K   A   H   G   I   D
ACA CTG GGC TTT GGT GCT TAC ATG TCC AAG GCT CAT GGG ATC GAT

                                                    260
 P   N   I   R   T   G   V   R   T   I   T   T   G   S   P
CCT AAC ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT GGC AGC CCC

                    270                             280
 I   T   Y   S   T   Y   G   K   F   L   A   D   G   G   C
ATC ACG TAC TCC ACC TAC GGC AAG TTC CTT GCC GAC GGC GGG TGC

                                                    290
 S   G   G   A   Y   D   I   I   I   C   D   E   C   H   S
TCG GGG GGC GCT TAT GAC ATA ATA ATT TGT GAC GAG TGC CAC TCC

                    300                             310
 T   D   A   T   S   I   L   G   I   G   T   V   L   D   Q
ACG GAT GCC ACA TCC ATC TTG GGC ATT GGC ACT GTC CTT GAC CAA

                                                    320
 A   E   T   A   G   A   R   L   V   V   L   A   T   A   T
GCA GAG ACT GCG GGG GCG AGA CTG GTT GTG CTC GCC ACC GCC ACC

                    330                             340
 P   P   G   S   V   T   V   P   H   P   N   I   E   E   V
CCT CCG GGC TCC GTC ACT GTG CCC CAT CCC AAC ATC GAG GAG GTT

                                                    350
 A   L   S   T   T   G   E   I   P   F   Y   G   K   A   I
GCT CTG TCC ACC ACC GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC

                    360                             370
 P   L   E   V   I   K   G   G   R   H   L   I   F   C   H
CCC CTC GAA GTA ATC AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT

                                                    380
 S   K   K   K   C   D   E   L   A   A   K   L   V   A   L
TCA AAG AAG AAG TGC GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG
```

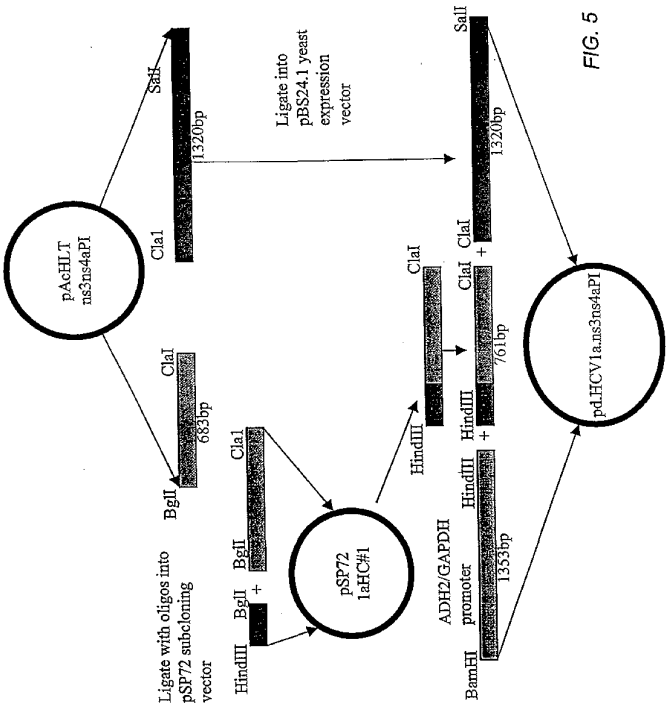
FIG. 4B

```
                390                                                 400
 G  I  N  A  V  A  Y  Y  R  G  L  D  V  S  V
GGC ATC AAT GCC GTG GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC

                                   410
 I  P  P  I  G  D  V  V  V  V  A  T  D  A  L
ATC CCG CCC ATC GGC GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC

                420                                                 430
 M  T  G  Y  T  G  D  F  D  S  V  I  D  C  N
ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT

                                   440
 T  C  V  T  Q  T  V  D  F  S  L  D  P  T  F
ACG TGT GTC ACC CAG ACA GTC GAT TTC AGC CTT GAC CCT ACC TTC

                450                                                 460
 T  I  E  T  I  T  L  P  Q  D  A  V  S  R  T
ACC ATT GAG ACA ATC ACG CTC CCC CAA GAT GCT GTC TCC CGC ACT

                                   470
 Q  R  R  G  R  T  G  R  G  K  P  G  I  Y  R
CAA CGT CGG GGC AGG ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA

                480                                                 490
 F  V  A  P  G  E  R  P  S  G  M  F  D  S  S
TTT GTG GCA CCG GGG GAG CGC CCC TCC GGC ATG TTC GAC TCG TCC

                                   500
 V  L  C  E  C  Y  D  A  G  C  A  W  Y  E  L
GTC CTC TGT GAG TGC TAT GAC GCA GGC TGT GCT TGG TAT GAG CTC

                510                                                 520
 T  P  A  E  T  T  V  R  L  R  A  Y  M  N  T
ACG CCC GCC GAG ACT ACA GTT AGG CTA CGA GCG TAC ATG AAC ACC

                                   530
 P  G  L  P  V  C  Q  D  H  L  E  F  W  E  G
CCG GGG CTT CCC GTG TGC CAG GAC CAT CTT GAA TTT TGG GAG GGC

                540                                                 550
 V  F  T  G  L  T  H  I  D  A  H  F  L  S  Q
GTC TTT ACA GGC CTC ACT CAT ATA GAT GCC CAC TTT CTA TCC CAG

                                   560
 T  K  Q  S  G  E  N  L  P  Y  L  V  A  Y  Q
ACA AAG CAG AGT GGG GAG AAC CTT CCT TAC CTG GTA GCG TAC CAA

                570                                                 580
 A  T  V  C  A  R  A  Q  A  P  P  P  S  W  D
GCC ACC GTG TGC GCT AGG GCT CAA GCC CCT CCC CCA TCG TGG GAC
```

FIG. 4C

```
                                                590
 Q   M   W   K   C   L   I   R   L   K   P   T   L   H   G
CAG ATG TGG AAG TGT TTG ATT CGC CTC AAG CCC ACC CTC CAT GGG

                    600                             610
 P   T   P   L   L   Y   R   L   G   A   V   Q   N   E   I
CCA ACA CCC CTG CTA TAC AGA CTG GGC GCT GTT CAG AAT GAA ATC


                                                620
 T   L   T   H   P   V   T   K   Y   I   M   T   C   M   S
ACC CTG ACG CAC CCA GTC ACC AAA TAC ATC ATG ACA TGC ATG TCG

                    630                             640
 A   D   L   E   V   V   T   S   T   W   V   L   V   G   G
GCC GAC CTG GAG GTC GTC ACG AGC ACC TGG GTG CTC GTT GGC GGC

                                                650
 V   L   A   A   L   A   A   Y   C   L   S   T   G   C   V
GTC CTG GCT GCT TTG GCC GCG TAT TGC CTG TCA ACA GGC TGC GTG

                    660                             670
 V   I   V   G   R   V   V   L   S   G   K   P   A   I   I
GTC ATA GTG GGC AGG GTC GTC TTG TCC GGG AAG CCG GCA ATC ATA

                                                680
 P   D   R   E   V   L   Y   R   E   F   D   E   M   E   E
CCT GAC AGG GAA GTC CTC TAC CGA GAG TTC GAT GAG ATG GAA GAG

686
 C
TGC
```

FIG. 4D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ns3ns4aPI
SalI
1320bp
ClaI
ClaI
683bp
BglII
Ligate with oligos into
pSP72 subcloning
vector
ClaI
BglII
BglII +
HindIII
pSP72
1aHC#1
Ligate into
pBS24.1 yeast
expression
vector
SalI
1320bp
ClaI
ClaI
HindIII
ClaI +
HindIII
761bp
HindIII +
ADH2/GAPDH
promoter
BamHI
1353bp
pdLHCV1a.ns3ns4aPI

FIG. 5

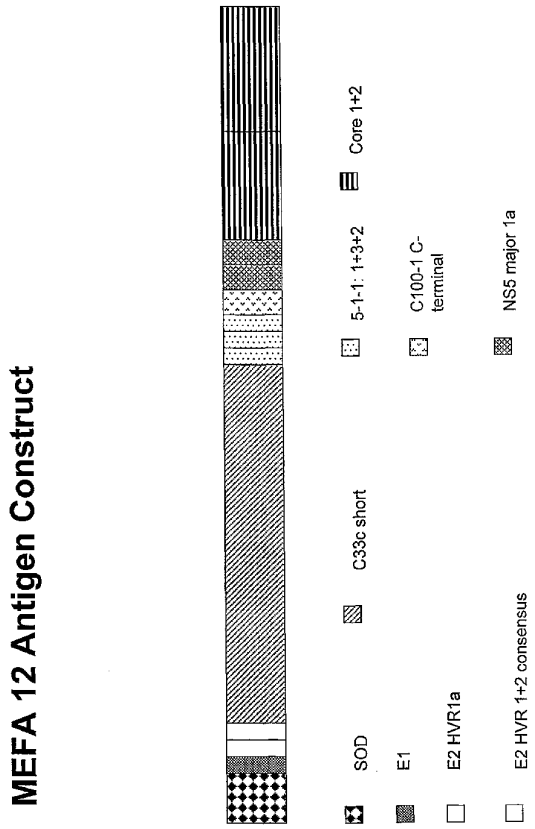
MEFA 12 Antigen Construct
SOD
C33c short
5-1-1: 1+3+2
Core 1+2
E1
E2 HVR1a
C100-1 C-terminal
NS5 major 1a
E2 HVR 1+2 consensus

FIG. 6

```
  1                                                  10
  M   A   T   K   A   V   C   V   L   K   G   D   G   P   V
ATG GCT ACA AAG GCT GTT TGT GTT TTG AAG GGT GAC GGC CCA GTT  45

                  20                                  30
  Q   G   I   I   N   F   E   Q   K   E   S   N   G   P   V
CAA GGT ATT ATT AAC TTC GAG CAG AAG GAA AGT AAT GGA CCA GTG  90

                                                  40
  K   V   W   G   S   I   K   G   L   T   E   G   L   H   G
AAG GTG TGG GGA AGC ATT AAA GGA CTG ACT GAA GGC CTG CAT GGA 135

                  50                                  60
  F   H   V   H   E   F   G   D   N   T   A   G   C   T   S
TTC CAT GTT CAT GAG TTT GGA GAT AAT ACA GCA GGC TGT ACC AGT 180

                                                  70
  A   G   P   H   F   N   P   L   S   T   R   G   C   N   C
GCA GGT CCT CAC TTT AAT CCT CTA TCC ACG CGT GGT TGC AAT TGC 225

                  80                                  90
  S   I   Y   P   G   H   I   T   G   H   R   M   A   W   K
TCT ATC TAT CCC GGC CAT ATA ACG GGT CAC CGC ATG GCA TGG AAG 270

                                                 100
  L   G   S   A   A   R   T   T   S   G   F   V   S   L   F
CTT GGT TCC GCC GCC AGA ACT ACC TCG GGC TTT GTC TCC TTG TTC 315

                 110                                 120
  A   P   G   A   K   Q   N   E   T   H   V   T   G   G   A
GCC CCA GGT GCC AAA CAA AAC GAA ACT CAC GTC ACG GGA GGC GCA 360

                                                 130
  A   A   R   T   T   S   G   L   T   S   L   F   S   P   G
GCC GCC CGA ACT ACG TCT GGG TTG ACC TCT TTG TTC TCC CCA GGT 405
```

FIG. 7A

```
                  140                                                 150
 A   S   Q   N    I   Q   L   I   T   S   T   D   N   S    S
GCC AGC CAA AAC ATT CAA TTG ATT ACT AGT ACG GAT AAC TCC TCT  450

                                      160
 P   P   V   V    P   Q   S   F   Q   V   A   H   L   H    A
CCA CCA GTA GTG CCC CAG AGC TTC CAG GTG GCT CAC CTC CAT GCT  495

                  170                                                 180
 P   T   G   S    G   K   S   T   K   V   P   A   A   Y    A
CCC ACA GGC AGC GGC AAA AGC ACC AAG GTC CCG GCT GCA TAT GCA  540

                                      190
 A   Q   G   Y    K   V   L   V   L   N   P   S   V   A    A
GCT CAG GGC TAT AAG GTG CTA GTA CTC AAC CCC TCT GTT GCT GCA  585

                  200                                                 210
 T   L   G   F    G   A   Y   M   S   K   A   H   G   I    D
ACA CTG GGC TTT GGT GCT TAC ATG TCC AAG GCT CAT GGG ATC GAT  630

                                      220
 P   N   I   R    T   G   V   R   T   I   T   T   G   S    P
CCT AAC ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT GGC AGC CCC  675

                  230                                                 240
 I   T   Y   S    T   Y   G   K   F   L   A   D   G   G    C
ATC ACG TAC TCC ACC TAC GGC AAG TTC CTT GCC GAC GGC GGG TGC  720

                                      250
 S   G   G   A    Y   D   I   I   I   C   D   E   C   H    S
TCG GGG GGC GCT TAT GAC ATA ATA ATT TGT GAC GAG TGC CAC TCC  765

                  260                                                 270
 T   D   A   T    S   I   L   G   I   G   T   V   L   D    Q
ACG GAT GCC ACA TCC ATC TTG GGC ATC GGC ACT GTC CTT GAC CAA  810

                                      280
 A   E   T   A    G   A   R   L   V   V   L   A   T   A    T
GCA GAG ACT GCG GGG GCG AGA CTG GTT GTG CTC GCC ACC GCC ACC  855

                  290                                                 300
 P   P   G   S    V   T   V   P   H   P   N   I   E   E    V
CCT CCG GGC TCC GTC ACT GTG CCC CAT CCC AAC ATC GAG GAG GTT  900
```

FIG. 7B

```
                                        310
 A   L   S   T   T   G   E   I   P   F   Y   G   K   A   I
GCT CTG TCC ACC ACC GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC  945

                320                                     330
 P   L   E   V   I   K   G   G   R   H   L   I   F   C   H
CCC CTC GAA GTA ATC AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT  990

                                        340
 S   K   K   K   C   D   E   L   A   A   K   L   V   A   L
TCA AAG AAG AAG TGC GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG 1035

                350                                     360
 G   I   N   A   V   A   Y   Y   R   G   L   D   V   S   V
GGC ATC AAT GCC GTG GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC 1080

                                        370
 I   P   T   S   G   D   V   V   V   V   A   T   D   A   L
ATC CCG ACC AGC GGC GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC 1125

                380                                     390
 M   T   G   Y   T   G   D   F   D   S   V   I   D   C   N
ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT 1170

                                        400
 T   C   A   C   S   G   K   P   A   I   I   P   D   R   E
ACG TGT GCA TGC TCC GGG AAG CCG GCA ATC ATA CCT GAC AGG GAA 1215

                410                                     420
 V   L   Y   R   E   F   D   E   M   E   E   C   S   Q   H
GTC CTC TAC CGA GAG TTC GAT GAG ATG GAA GAG TGC TCT CAG CAC 1260

                                        430
 L   P   Y   I   E   Q   G   M   M   L   A   E   Q   F   K
TTA CCG TAC ATC GAG CAA GGG ATG ATG CTC GCC GAG CAG TTC AAG 1305

                440                                     450
 Q   K   A   L   G   L   S   R   G   G   K   P   A   I   V
CAG AAG GCC CTC GGC CTC TCG CGA GGG GGC AAG CCG GCA ATC GTT 1350

                                        460
 P   D   K   E   V   L   Y   Q   Q   Y   D   E   M   E   E
CCA GAC AAA GAG GTG TTG TAT CAA CAA TAC GAT GAG ATG GAA GAG 1395
```

FIG. 7C

```
                  470                                                 480
 C   S   Q   A    A   P   Y   I   E   Q   A   Q   V   I   A
TGC TCA CAA GCT GCC CCA TAT ATC GAA CAA GCT CAG GTA ATA GCT  1440

                                      490
 H   Q   F   K    E   K   V   L   G   L   I   D   N   D   Q
CAC CAG TTC AAG GAA AAA GTC CTT GGA TTG ATC GAT AAT GAT CAA  1485

                  500                                                 510
 V   V   V   T    P   D   K   E   I   L   Y   E   A   F   D
GTG GTT GTG ACT CCT GAC AAA GAA ATC TTA TAT GAG GCC TTT GAT  1530

                                      520
 E   M   E   E    C   A   S   K   A   A   L   I   E   E   G
GAG ATG GAA GAA TGC GCC TCC AAA GCC GCC CTC ATT GAG GAA GGG  1575

                  530                                                 540
 Q   R   M   A    E   M   L   K   S   K   I   Q   G   L   L
CAG CGG ATG GCG GAG ATG CTC AAG TCT AAG ATA CAA GGC CTC CTC  1625

                                      550
 G   I   L   R    R   H   V   G   P   G   E   G   A   V   Q
GGG ATA CTG CGC CGG CAC GTT GGT CCT GGC GAG GGG GCA GTG CAG  1670

                  560                                                 570
 W   M   N   R    L   I   A   F   A   S   R   G   N   H   V
TGG ATG AAC CGG CTG ATA GCC TTC GCC TCC AGA GGG AAC CAT GTT  1715

                                      580
 S   P   T   H    Y   V   P   S   R   S   R   R   F   A   Q
TCC CCC ACG CAC TAC GTT CCG TCT AGA TCC CGG AGA TTC GCC CAG  1760

                  590                                                 600
 A   L   P   V    W   A   R   P   D   Y   N   P   P   L   V
GCC CTG CCC GTT TGG GCG CGG CCG GAC TAT AAC CCC CCG CTA GTG  1805

                                      610
 E   T   W   K    K   P   D   Y   E   P   P   V   V   H   G
GAG ACG TGG AAA AAG CCC GAC TAC GAA CCA CCT GTG GTC CAC GGC  1850

                  620                                                 630
 R   S   S   R    R   F   A   Q   A   L   P   V   W   A   R
AGA TCT TCT CGG AGA TTC GCC CAG GCC CTG CCC GTT TGG GCG CGG  1895
```

FIG. 7D

```
                                        640
 P   D   Y   N   P   P   L   V   E   T   W   K   K   P   D
CCG GAC TAT AAC CCC CCG CTA GTG GAG ACG TGG AAA AAG CCC GAC  1940

                650                                     660
 Y   E   P   P   V   V   H   G   R   K   T   K   R   N   T
TAC GAA CCA CCT GTG GTC CAT GGC AGA AAG ACC AAA CGT AAC ACC  1985

                                        670
 N   R   R   P   Q   D   V   K   F   P   G   G   G   Q   I
AAC CGG CGG CCG CAG GAC GTC AAG TTC CCG GGT GGC GGT CAG ATC  2030

                680                                     690
 V   G   G   V   Y   L   L   P   R   R   G   P   R   L   G
GTT GGT GGA GTT TAC TTG TTG CCG CGC AGG GGC CCT AGA TTG GGT  2075

                                        700
 V   L   A   T   R   K   T   S   P   I   P   K   A   R   R
GTG CTC GCG ACG AGA AAG ACT TCC CCT ATC CCC AAG GCT CGT CGG  2120

                710                                     720
 P   E   G   R   T   W   A   Q   P   G   Y   P   W   P   L
CCC GAG GGC AGG ACC TGG GCT CAG CCC GGT TAC CCT TGG CCC CTC  2165

                                        730
 Y   G   N   K   D   R   R   S   T   G   K   S   W   G   K
TAT GGC AAT AAG GAC AGA CGG TCT ACA GGT AAG TCC TGG GGT AAG  2210

                740                                     750
 P   G   Y   P   W   P   R   K   T   K   R   N   T   N   R
CCA GGG TAC CCT TGG CCA AGA AAG ACC AAA CGT AAC ACC AAC CGG  2255

                                        760
 R   P   Q   D   V   K   F   P   G   G   G   Q   I   V   G
CGG CCG CAG GAC GTC AAG TTC CCG GGT GGC GGT CAG ATC GTT GGT  2300

                770                                     780
 G   V   Y   L   L   P   R   R   G   P   R   L   G   V   L
GGA GTT TAC TTG TTG CCG CGC AGG GGC CCT AGA TTG GGT GTG CTC  2345

                                        790
 A   T   R   K   T   S   P   I   P   K   A   R   R   P   E
GCG ACG AGA AAG ACT TCC CCT ATC CCC AAG GCT CGT CGG CCC GAG  2390
```

FIG. 7E

```
                  800                                                 810
 G   R   T   W   A   Q   P   G   Y   P   W   P   L   Y   G
GGC AGG ACC TGG GCT CAG CCC GGT TAC CCT TGG CCC CTC TAT GGC  2435

                                          820
 N   K   D   R   R   S   T   G   K   S   W   G   K   P   G
AAT AAG GAC AGA CGG TCT ACA GGT AAG TCC TGG GGT AAG CCA GGG  2480

              829
 Y   P   W   P   OC
TAC CCT TGG CCC TAA TGAGTCGAC
```

FIG. 7F

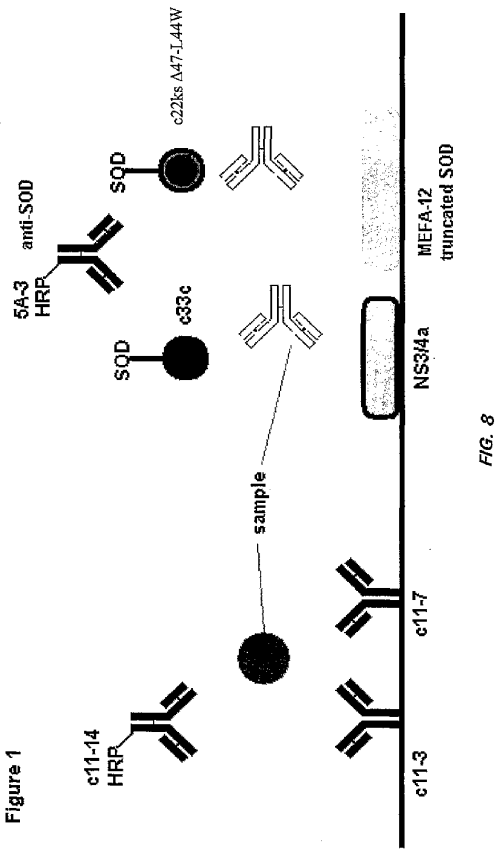

Figure 1

FIG. 8

【国際公開パンフレット（コレクトバージョン）】

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(19) World Intellectual Property Organization
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(75) Inventors/Applicants (*for US only*): **CHIEN, David, Y.** [US/US]; 1121 Douglas Court, Alamo, CA 94507 (US). **ARCANGEL, Phillip** [US/US]; 567 Oakland Avenue, #310, Oakland, CA 94611 (US). **TANDESKE, Laura** [US/US]; 305 Melven Court, San Leandro, CA 94577 (US). **GEORGE-NASCIEMENTO, Carlos** [CL/US]; 2061 Magnolia Way, Walnut Creek, CA 94595 (US). **COIT, Doris** [US/US]; 1058 Rancho Lindo Drive, Petaluma, CA 94952 (US). **MEDINA-SELBY, Angelica** [CL/US]; 136 Galewood Circle, San Francisco, CA 94131 (US).

(74) Agents: **HARBIN, Alisa, A.**; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662 et al. (US).

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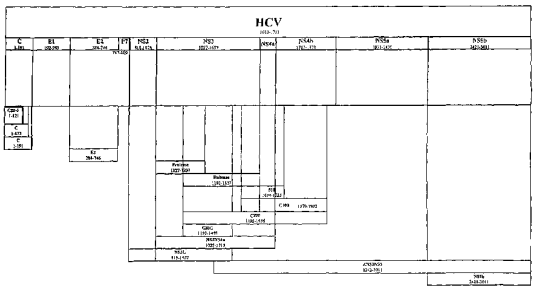
[Continued on next page]

(54) Title: HCV ANTIGEN/ANTIBODY COMBINATION ASSAY

HCV Genome and Recombinant Proteins



(57) Abstract: An HCV core antigen and NS3/4a antibody combination assay that can detect both HCV antigens and antibodies present in a sample using a single solid matrix, is provided, as well as immunoassay solid supports for use in the assay.

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【国際調査報告】

INTERNATIONAL SEARCH REPORT

In ıl Application No
PCT/US '01/19369

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KASHIWAKUMA T ET AL: "Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA)" JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 190, no. 1, 28 March 1996 (1996-03-28), pages 79-89, XP004020891 ISSN: 0022-1759 the whole document --- -/--	1-39

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
5 March 2003	14/04/03

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hinchliffe, P

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INTERNATIONAL SEARCH REPORT

Ir al Application No
PCT/US 01/19369

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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摘要(译)

根据本发明，提供了HCV核心抗原和NS3 / 4a抗体的组合测定。该组合的HCV核心抗原和NS3 / 4a抗体测定可以使用单个固体基质检测样品中存在的HCV抗原和HCV抗体。此外，根据本发明，还提供了用于HCV核心抗原和NS3 / 4a抗体的该组合测定的免疫测定固体支持物。