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(54) **IMMUNOASSAYS AND KITS FOR THE  
DETECTION OF NGAL**

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(75) Inventors: **Larry G. Birkenmeyer**, Glenview,  
IL (US); **Suresh M. Desai**,  
Libertyville, IL (US); **Frank C.  
Grenier**, Libertyville, IL (US);  
**David J. Hawksworth**, Lake Villa,  
IL (US); **Edward T. Olejniczak**,  
Grayslake, IL (US); **Qiaoqiao  
Ruan**, Round Lake, IL (US);  
**Robert W. Siegel**, Fountaintown,  
IN (US); **Sergey Y. Tetin**,  
Lindenhurst, IL (US); **Bryan C.  
Tieman**, Elmhurst, IL (US); **Bailin  
Tu**, Libertyville, IL (US); **Joan D.  
Tyner**, Beach Park, IL (US); **Ryan  
F. Workman**, Gurnee, IL (US);  
**Robert N. Ziemann**, Lindenhurst,  
IL (US)

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Correspondence Address:

**PAUL D. YASGER**  
**ABBOTT LABORATORIES**  
**100 ABBOTT PARK ROAD, DEPT. 377/AP6A**  
**ABBOTT PARK, IL 60064-6008 (US)**

(57) **ABSTRACT**

The present invention relates to NGAL immunoassays and kits, and to methods of using glycosylated mammalian NGAL and antibodies that bind to mammalian NGAL in immunoassays and kits. Among other things, the methods and kits can be employed to determine the amount of human NGAL monomer in a test sample, as well as to determine the proportion of human NGAL monomer to human NGAL dimer contained in a test sample.

(73) Assignee: **ABBOTT LABORATORIES**,  
Abbott Park, IL (US)

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Signal Peptide

MPLGLLWLGL ALLGALHAQA QDSTSDLIPA PPLSKVPLQQ  
NFQDNQFQGK WYVVGLAGNA ILREDKDPQK MYATIYELKE  
DKSYNVTSVL FRKKKCDYWI RTFVPGCQPG EFTLGNIKSY  
PGLTSYLVRV VSTNYNQHAM VFFKKVSQNR EYFKITLYGR  
TKELTSELKE NFIRFSKSLG LPENHIVFPV PIDQCIDGHH

HHHH

6X His Tag

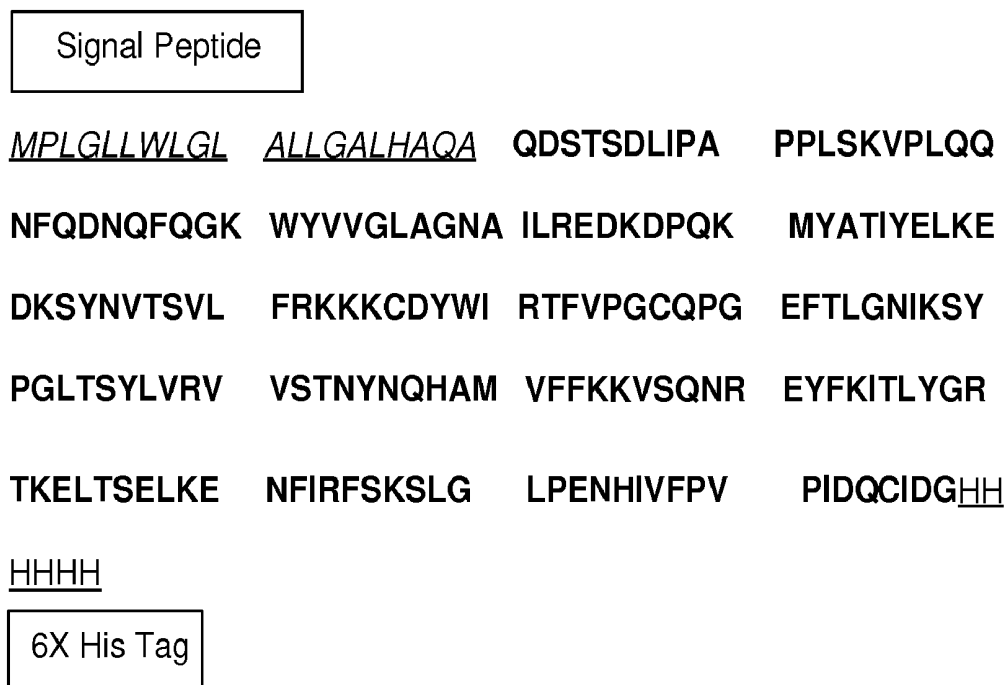


Figure 1

## IMMUNOASSAYS AND KITS FOR THE DETECTION OF NGAL

### RELATED APPLICATION INFORMATION

**[0001]** This application claims the priority of U.S. Provisional Application Ser. Nos. 60/981,470, 60/981,471 and 60/981,473, all filed on Oct. 19, 2007 (now all expired), is a continuation-in-part application of U.S. Nonprovisional application Ser. Nos. 12/104,408, 12/104,410, and 12/104,413, all filed on Apr. 16, 2008 (all pending), and is a continuation application of PCT International Application PCT/US08/80331 filed Oct. 17, 2008 (pending), all of which are incorporated by reference in their entireties.

### TECHNICAL FIELD

**[0002]** The present invention relates to NGAL immunoassays and kits, and to methods of using glycosylated mammalian NGAL and antibodies that bind to mammalian NGAL in immunoassays and kits. Such immunoassays and kits among other things optionally provide for improved detection of monomer. The methods and kits can be employed to determine the amount of human NGAL monomer in a test sample, as well as to determine the proportion of human NGAL monomer to human NGAL dimer contained in a test sample.

### BACKGROUND

**[0003]** Lipocalins are a family of extracellular ligand-binding proteins that are found in a variety of organisms from bacteria to humans. Lipocalins possess many different functions, such as the binding and transport of small hydrophobic molecules, nutrient transport, cell growth regulation, and modulation of the immune response, inflammation and prostaglandin synthesis. Moreover, some lipocalins are also involved in cell regulatory processes and serve as diagnostic and prognostic markers in a variety of disease states. For example, the plasma level of alpha glycoprotein is monitored during pregnancy and in diagnosis and prognosis of conditions including cancer chemotherapy, renal dysfunction, myocardial infarction, arthritis, and multiple sclerosis.

**[0004]** The novel lipocalin neutrophil gelatinase-associated lipocalin (or NGAL, also known as Lipocalin-2 or LCN2) from human neutrophils was identified in 1993. NGAL is a 25-kDa lipocalin that exists in monomeric and homo- and heterodimeric forms, the latter as a 46-kDa dimer with human neutrophil gelatinase. A trimer form of NGAL has also been identified. NGAL is secreted from specific granules of activated human neutrophils. Homologous proteins have been identified in mouse (24p3/uterocalin) and rat ( $\alpha$  (2)-microglobulin-related protein/neu-related lipocalin). Structural data have confirmed a typical lipocalin fold of NGAL with an eight-stranded beta-barrel, but with an unusually large cavity lined with more polar and positively charged amino acid residues than normally seen in lipocalins. The 25-kDa NGAL protein is believed to bind small lipophilic substances such as bacteria-derived lipopolysaccharides and formylpeptides, and may function as a modulator of inflammation.

**[0005]** Renal injuries or disease, such as acute kidney failure or chronic kidney failure, can result from a variety of different causes (such as illness, injury, and the like). The early identification and treatment of renal injuries and disease would be useful in preventing disease progression. Currently, serum creatinine is frequently used as a biomarker of kidney

function. However, serum creatinine measurements are influenced by muscle mass, gender, race and medications. Unfortunately, these limitations often result in the diagnosis of kidney disease only after significant damage has already occurred.

**[0006]** NGAL is an early marker for acute renal injury or disease. In addition to being produced by specific granules of activated human neutrophils, NGAL is also produced by nephrons in response to tubular epithelial damage and is a marker of tubulointerstitial (TI) injury. NGAL levels rise in acute tubular necrosis (ATN) from ischemia or nephrotoxicity, even after mild "subclinical" renal ischemia, as compared to normal serum creatinine levels. Moreover, NGAL is known to be expressed by the kidney in cases of chronic kidney disease (CKD). Elevated urinary NGAL levels have been suggested as predictive of progressive kidney failure. It has been previously demonstrated that NGAL is markedly expressed by kidney tubules very early after ischemic or nephrotoxic injury in both animal and human models. NGAL is rapidly secreted into the urine, where it can be easily detected and measured, and precedes the appearance of any other known urinary or serum markers of ischemic injury. The protein is resistant to proteases, suggesting that it can be recovered in the urine as a faithful marker of tubule expression of NGAL. Further, NGAL derived from outside of the kidney, for example, filtered from the blood, does not appear in the urine, but rather is quantitatively taken up by the proximal tubule.

**[0007]** A variety of immunoassays are known in the art for detecting NGAL. As mentioned previously herein, NGAL is found as a monomer, as a dimer (a homodimer or heterodimer) and even as a trimer. Thus, there is a need in the art for new antibodies and immunoassays which are able to specifically detect and distinguish between NGAL monomer, dimer or trimer in a test sample. Additionally, there is also a need in the art for immunoassays that are able to quantify the relative proportion of monomer to dimer contained in a test sample. Such new antibodies and immunoassays can be used to assess among other things the extent of any renal injury or disease in a patient, monitor the kidney status of a patient suffering from renal injury or disease, or assess the extent of any renal injury in a patient and thereafter monitor the patient's kidney status. Additional objects and advantages of the invention will be apparent from the description provided herein.

### SUMMARY

**[0008]** In one embodiment, the present invention relates to a method for determining the amount of human NGAL monomer in a test sample. The method comprising the steps of:

**[0009]** (a) contacting a test sample suspected of containing human NGAL monomer and human NGAL dimer with at least one first antibody (e.g., a capture antibody) so as to form a first antibody/human NGAL complex, wherein the at least one capture first antibody binds to human NGAL and is an antibody (e.g., a capture antibody) selected from the group consisting of an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026;

**[0010]** (b) contacting the antibody/human NGAL complex with a second antibody that binds to human NGAL and that has been conjugated to a detectable label to form a second antibody/human NGAL/first antibody complex, wherein the

second antibody differs from the first antibody and is an antibody selected from the group consisting of: an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026; and

**[0011]** (c) determining with at least about 75% specificity the amount of human NGAL monomer contained in the test sample based on the amount of the second antibody/human NGAL/first antibody complex formed in step (b).

**[0012]** In the above method, the test sample is urine or blood. Specifically, the test sample is urine.

**[0013]** In the above method, the method is carried out to evaluate the renal tubular cell injury status of the subject based on the level of NGAL present in the test sample. Specifically, the renal tubular cell injury comprises an injury selected from the group consisting of an ischemic renal injury, a nephrotoxic injury, and any other injury that affects the tubular cells of the kidney.

**[0014]** In the above method, at least one first antibody is immobilized on a solid phase either prior to or following contacting with the test sample. Optionally, the at least one first antibody is immobilized on a solid phase prior to formation of the second antibody/human NGAL/first antibody complex. Optionally, the at least one first antibody is immobilized on a solid phase prior to formation of the first antibody/human NGAL complex. Optionally, at least one first antibody is immobilized on a solid phase after formation of the first antibody/human NGAL complex.

**[0015]** In one aspect, the above method is performed in the absence of any reducing agents. Alternatively, in the above method, steps (a), (b) and (c) are performed in the presence of or following treatment of the test sample with at least one reducing agent. The at least one reducing agent is selected from the group consisting of dithiothreitol, 2-mercaptoethanol, 2-mercaptoethylamine, and Tris(2-carboxyethyl)phosphine. The at least one reducing agent is present in an amount of from about 0.1 mM to about 500 mM, especially an amount of from about 0.1 mM to about 100 mM. In another aspect, the method is adapted for use in an automated system or semi-automated system.

**[0016]** The detectable label used in the above method is selected from the group consisting of a radioactive label, an enzymatic label, a chemiluminescent label, a fluorescence label, a thermometric label, and an immuno-polymerase chain reaction label. Specifically, the detectable label is acridinium. In particular, the method optimally is about four times as specific for NGAL monomer as compared to NGAL dimer throughout the calibration range of the assay (e.g., from about 1 ng/mL up to about 2000 ng/ml of NGAL, especially urinary NGAL).

**[0017]** In another embodiment, the present invention relates to a method for determining the proportion of human NGAL monomer to human NGAL dimer contained in a test sample. The method optionally comprises the steps of:

**[0018]** (a) contacting a test sample suspected of containing human NGAL monomer and human NGAL dimer with at least one first antibody so as to form a first antibody/human NGAL complex, wherein the at least one first antibody binds to human NGAL and is an antibody selected from the group consisting of an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026;

**[0019]** (b) contacting the first antibody/human NGAL complex with a second antibody that binds to human NGAL and that has been conjugated to a detectable label so as to form a second antibody/human NGAL/first antibody complex, wherein the second antibody differs from the first antibody and is an antibody selected from the group consisting of an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026;

**[0020]** (c) determining the amount of the second antibody/human NGAL/first antibody complex formed in step (b), wherein steps (a) and (b) are performed in the absence of any reducing agents;

**[0021]** (d) determining the amount of the second antibody/human NGAL complex formed in step (b), wherein steps (a) and (b) are performed in the presence of or following treatment of the test sample with at least one reducing agent; and

**[0022]** (e) determining the proportion of human NGAL monomer to human NGAL dimer in the test sample based on comparing the amount of the second antibody/human NGAL/first antibody complex determined in step (c) and the amount of the second antibody/human NGAL/first antibody complex determined in step (d).

**[0023]** In the above method, the at least one first antibody optionally is immobilized on a solid phase either prior to or following contacting with the test sample. Optionally, the at least one first antibody is immobilized on a solid phase prior to formation of the second antibody/human NGAL/first antibody complex. Optionally, the at least one first antibody is immobilized on a solid phase prior to formation of the first antibody/human NGAL complex. In yet another embodiment, optionally, the at least one first antibody is immobilized on a solid phase after formation of the first antibody/human NGAL complex. The at least one reducing agent is selected from the group consisting of dithiothreitol, 2-mercaptoethanol, 2-mercaptoethylamine, and Tris(2-carboxyethyl)phosphine. The at least one reducing agent is present in the amount of from about 0.1 mM to about 500 mM, especially an amount of from about 0.1 mM to about 100 mM. Optionally the above method is adapted for use in an automated system or semi-automated system.

**[0024]** The detectable label used in the above method is selected from the group consisting of a radioactive label, an enzymatic label, a chemiluminescent label, a fluorescence label, a thermometric label, and an immuno-polymerase chain reaction label. Specifically, the detectable label is acridinium.

**[0025]** In the above method, the test sample is urine or blood. Specifically, the test sample is urine.

**[0026]** In the above method, the method is carried out to evaluate the renal tubular cell injury status of the subject based on the level of NGAL present in the test sample. Specifically, the renal tubular cell injury comprises an injury selected from the group consisting of an ischemic renal injury, a nephrotoxic injury, and an other injury that affects the tubular cells of the kidney.

**[0027]** In another embodiment, the present invention relates to an improvement of a method for detecting the presence of mammalian NGAL in a test sample. The method comprises the steps of:

**[0028]** (a) contacting a test sample suspected of containing mammalian NGAL with at least one antibody specific for the

mammalian NGAL for a time and under conditions that allow the formation of a mammalian NGAL/antibody complex; and [0029] (b) detecting any mammalian NGAL/antibody complex formed as indicating the presence of the mammalian NGAL;

[0030] wherein the improvement comprises employing as a calibrator or control the calibrator or control as described previously herein, particularly wherein the calibrator or control is glycosylated human NGAL comprising the sequence of SEQ ID NOS:1 or 13.

[0031] In yet another embodiment, the present invention relates to a diagnostic kit for the detection of mammalian NGAL comprising a calibrator or control as described herein, particularly where the calibrator or control selected from the group consisting of:

[0032] (a) glycosylated human NGAL comprising the sequence of SEQ ID NOS:2 or 10, and

[0033] (b) glycosylated human NGAL comprising the sequence of SEQ ID NOS:1 or 13.

[0034] In another embodiment, the present invention relates to an improvement of a method for detecting the presence of method for detecting the presence of human NGAL antigen in a test sample, wherein the method comprises:

[0035] (a) contacting a test sample suspected of containing human NGAL with an immunodiagnostic reagent as set forth herein for a time and under conditions that allow formation of a human NGAL/antibody complex; and

[0036] (b) detecting any human NGAL/antibody complex formed as indicating the presence of the human NGAL antigen.

[0037] Generally, as discussed further herein an immunodiagnostic reagent comprises one or more antibodies selected from the group consisting of:

[0038] (a) an antibody that specifically binds to a conformational epitope comprising amino acid residues 112, 118 and 147 of human NGAL protein as set forth in SEQ ID NOS:1, 2, 10 or 13;

[0039] (b) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5;

[0040] (c) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0041] (d) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0042] (e) an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024;

[0043] (f) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:7;

[0044] (g) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:8;

[0045] (h) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ

ID NO:7 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:8; and

[0046] (i) an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026.

[0047] The invention further accordingly provides a diagnostic kit for the detection of human NGAL comprising the aforementioned immunodiagnostic reagent, and instructions.

#### BRIEF DESCRIPTION OF THE FIGURE

[0048] FIG. 1 shows the human NGAL wild-type antigen sequence (SEQ ID NO:1). Native human NGAL signal peptide residues are in italics and underlined. Wild-type human NGAL sequences in pJV-NGAL-A3 plasmid are in bold. The 6×His tag in the C-terminal is underlined.

#### DETAILED DESCRIPTION

[0049] Glycosylated mammalian NGAL proteins, and antibodies that bind to mammalian NGAL proteins have been discovered. These NGAL proteins and antibodies alone or in or in combination have a variety of uses, for example, as a component of a diagnostic assay, or present in an immunoassay kit.

[0050] All NGAL polynucleotide and polypeptide sequences, and wild-type NGAL recombinant antigen (rAg) and mutant C87S NGAL NGAL rAg clones, subclones, hybrids, and hybridomas (including names and numbering) are as described in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

[0051] Antibodies that bind to certain mammalian NGAL proteins also have been discovered. These anti-NGAL antibodies (also loosely referred to herein as “NGAL antibodies”), are as described in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

#### A. DEFINITIONS

[0052] As used herein, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the numbers 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9 and 7.0 are explicitly contemplated.

##### a) Antibody

[0053] As used herein, the terms “antibody” and “antibodies” refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies (in one aspect, a bird (for example, a duck or goose), in another aspect, a shark or whale, in yet another aspect, a mammal, including a non-primate (for example, a cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, feline, canine, rat, murine, etc) and a non-human primate (for example, a monkey, such as a cynomologous monkey, a chimpanzee, etc), recombinant antibodies, chimeric antibodies, single-chain Fvs (scFv), single chain antibodies, single domain antibodies, Fab fragments, F(ab)<sub>2</sub> fragments, disulfide-linked Fv (sdFv), and anti-idiotypic (anti-Id) antibodies (including, for example, anti-Id antibodies to antibodies of the present inven-

tion), and functionally active epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, namely, molecules that contain an antigen binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA and IgY), class (for example, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub> and IgA<sub>2</sub>) or subclass. For simplicity sake, an antibody against an analyte is frequently referred to as being either an “anti-analyte antibody”, or merely an “analyte antibody” (e.g., an NGAL antibody).

#### b) Renal Tubular Cell Injury

**[0054]** As used herein the expression “renal tubular cell injury” means a renal or kidney failure or dysfunction, either sudden (acute) or slowly declining over time (chronic), that can be triggered by a number of disease or disorder processes. Both acute and chronic forms of renal tubular cell injury can result in a life-threatening metabolic derangement.

#### c) Acute Kidney Disease

**[0055]** An “acute renal tubular cell injury” means acute ischemic renal injury (IRI) or acute nephrotoxic renal injury (NRI). IRI includes but is not limited to ischemic injury and chronic ischemic injury, acute renal failure, acute glomerulonephritis, and acute tubulo-interstitial nephropathy. NRI toxicity includes but is not limited to, sepsis (infection), shock, trauma, kidney stones, kidney infection, drug toxicity, poisons or toxins, or after injection with a radiocontrast dye.

#### d) Chronic Kidney Disease

**[0056]** The phrases “chronic renal tubular cell injury”, “progressive renal disease”, “chronic renal disease (CRD)”, “chronic kidney disease (CKD)” as used interchangeably herein, include any kidney condition or dysfunction that occurs over a period of time, as opposed to a sudden event, to cause a gradual decrease of renal tubular cell function or worsening of renal tubular cell injury. One endpoint on the continuum of chronic renal disease is “chronic renal failure (CRF)”. For example, chronic kidney disease or chronic renal injury as used interchangeably herein, includes, but is not limited to, conditions or dysfunctions caused by chronic infections, chronic inflammation, glomerulonephritides, vascular diseases, interstitial nephritis, drugs, toxins, trauma, renal stones, long standing hypertension, diabetes, congestive heart failure, nephropathy from sickle cell anemia and other blood dyscrasias, nephropathy related to hepatitis, HIV, parvovirus and BK virus (a human polyomavirus), cystic kidney diseases, congenital malformations, obstruction, malignancy, kidney disease of indeterminate causes, lupus nephritis, membranous glomerulonephritis, membranoproliferative glomerulonephritis, focal glomerular sclerosis, minimal change disease, cryoglobulinemia, Anti-Neutrophil Cytoplasmic Antibody (ANCA)-positive vasculitis, ANCA-negative vasculitis, amyloidosis, multiple myeloma, light chain deposition disease, complications of kidney transplant, chronic rejection of a kidney transplant, chronic allograft nephropathy, and the chronic effects of immunosuppressives. Preferably, chronic renal disease or chronic renal injury refers to chronic renal failure or chronic glomerulonephritis.

#### e) Immunodiagnostic Reagent

**[0057]** An “immunodiagnostic reagent” according to the present invention comprises one or more antibodies that spe-

cifically bind to a region of an NGAL protein. Immunodiagnostic reagents are as set forth in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

#### f) NGAL Polynucleotide and Polypeptide Sequences

**[0058]** NGAL polynucleotide and polypeptide sequences are as described in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same). Generally, the NGAL can be any NGAL sequence, e.g., including that set forth as Genbank accession numbers Genpept CAA58127 (SEQ ID NO:1), AAB26529, XP\_862322, XP\_548441, P80108, P11672, X83006.1, X99133.1, CAA67574.1, BC033089.1, AAH33089.1, 575256.1, AD14168.1, JC2339, 1DFVA, 1DFVB, 1L6MA, 1L6 MB, 1L6MC, 1NGLA, 1QQSA, 1X71A, 1X71B, 1X71C, 1X89A, 1X89B, 1X89C, 1X8UA, 1X8UB, and 1X8UC. NGAL polynucleotide and polypeptide (e.g., polyamino acid) sequences are as found in nature, based on sequences found in nature, isolated, synthetic, semi-synthetic, recombinant, or other. In one embodiment, the NGAL is human NGAL (also known as “hNGAL”). Unless specified otherwise, NGAL polypeptide sequences are numbered according to the mature human NGAL sequence minus the 20 residue amino acid signal peptide typically found in nature (and minus any other signal peptide sequence). When a signal peptide is present, it is numbered with negative numbers, e.g., as residues -1 to -20, with comparable numbering applied for the encoding polynucleotide sequence.

**[0059]** Likewise, an initial Met residue at the N-terminus of NGAL is present only in NGAL produced in prokaryotes (e.g., *E. coli*), or in synthetic (including semi-synthetic) or derived sequences, and not in NGAL produced in eukaryotes (e.g., mammalian cells, including human and yeast cells). Consequently, when present, an initial Met residue is counted herein as a negative number, e.g., as residue -1, with no similar numbering adjustment being made for the polynucleotide sequence in a prokaryotic versus eukaryotic background or expression system inasmuch as the polynucleotide sequence is replicated and transcribed the same in both backgrounds and the difference lies at the level of translation.

**[0060]** Accordingly, the disclosure herein encompasses a multitude of different NGAL polynucleotide and polypeptide sequences as present and/or produced in a prokaryotic and/or eukaryotic background (e.g., with consequent optimization for codon recognition). In sum, the sequences may or may not possess or encode: (a) a signal peptide; (b) an initiator Met residue present in the mature NGAL sequence at the N-terminus; (c) an initiator Met residue present at the start of a signal peptide that precedes the mature NGAL protein; and (d) other variations such as would be apparent to one skilled in the art.

**[0061]** Exemplary sequences include, but are not limited to, those as set forth herein: SEQ ID NO:1 (NGAL wild-type polypeptide including signal peptide); SEQ ID NO:2 (NGAL mutant polypeptide including signal peptide); SEQ ID NO:10 (NGAL mutant polypeptide not including any signal peptide, and which can be preceded by a Met initiator residue when produced in prokaryotes and a Met initiator codon is present; however, there is no Met initiator residue when produced in eukaryotes, regardless of whether a Met initiator codon is present); SEQ ID NO:13 (NGAL wild-type polypeptide not including any signal peptide, and which can be preceded by a Met initiator residue when produced in prokaryotes and a Met

initiator codon is present; however, there is no Met initiator residue when produced in eukaryotes, regardless of whether a Met initiator codon is present); SEQ ID NO:3 (NGAL wild-type polynucleotide sequence including that encoding a signal peptide); SEQ ID NO:4 (NGAL mutant polynucleotide including that encoding a signal peptide); SEQ ID NO:12 (NGAL mutant polynucleotide, synthetic or for eukaryotic expression, not including that encoding any signal peptide, but which optionally further can be preceded at the N-terminus either with or without a Met initiator codon, e.g., ATG); SEQ ID NO:9 (NGAL mutant polynucleotide, synthetic or for prokaryotic expression, not including that encoding any signal peptide, but which optionally further can be preceded at the N-terminus either with or without a Met initiator codon, e.g., ATG).

#### g) Glycosylated Mammalian NGAL

**[0062]** Glycosylated mammalian NGAL (e.g., employed as an immunogen and/or assessing the binding of various antibodies) is as described in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

**[0063]** Generally, as used herein, the phrases “oligosaccharide moiety” or “oligosaccharide molecule” as used interchangeably herein refers to a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to a polypeptide (to produce a glycosylated polypeptide, such as, for example, mammalian NGAL) by way of *in vivo* or *in vitro* glycosylation. Except where the number of oligosaccharide moieties attached to the polypeptide is expressly indicated, every reference to “oligosaccharide moiety” referred to herein is intended as a reference to one or more such moieties attached to a polypeptide. Preferably, the polypeptide to which said carbohydrate-containing molecule is capable of being attached is wild-type or mutant mammalian NGAL, i.e., to provide “glycosylated mammalian NGAL” as described further herein.

**[0064]** The term “*in vivo* glycosylation” is intended to mean any attachment of an oligosaccharide moiety occurring *in vivo*, for example, during posttranslational processing in a glycosylating cell used for expression of the polypeptide, for example, by way of N-linked and O-linked glycosylation. Usually, the N-glycosylated oligosaccharide-moiety has a common basic core structure composed of five monosaccharide residues, namely two N-acetylglucosamine residues and three mannose residues. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question and on the specific polypeptide.

**[0065]** The phrase “*in vitro* glycosylation” refers to a synthetic glycosylation performed *in vitro*, normally involving covalently linking an oligosaccharide moiety to an attachment group of a polypeptide, optionally using a cross-linking agent. *In vitro* glycosylation can be achieved by attaching chemically synthesized oligosaccharide structures to a polypeptide (such as, for example, mammalian NGAL) using a variety of different chemistries. For example, the chemistries that can be employed are those used for the attachment of polyethylene glycol (PEG) to proteins, wherein the oligosaccharide is linked to a functional group, optionally, via a short spacer. *In vitro* glycosylation can be carried out in a suitable buffer at a pH of about 4.0 to about 7.0 in protein concentrations of about 0.5 to about 2.0 mg/mL in a volume of about 0.02 to about 2.0 ml. Other *in vitro* glycosylation methods are described, for example in WO 87/05330, by Aplin et al., *CRC*

*Crit. Rev. Biochem.* 259-306 (1981), by Lundblad et al. in *Chemical Reagents for Protein Modification*, CRC Press Inc., Boca Raton, Fla., Yan et al., *Biochemistry*, 23:3759-3765 (1982) and Doebber et al., *J. Biol. Chem.*, 257:2193-2199 (1982).

#### h) Human NGAL Fragment

**[0066]** A human NGAL fragment (e.g., employed as an immunogen, calibrator, control and/or for assessing the binding of various antibodies) is as described in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

**[0067]** Generally, as used herein, the term “human NGAL fragment” herein refers to a polypeptide that comprises a part that is less than the entirety of a mature human NGAL or NGAL including a signal peptide. In particular, a human NGAL fragment comprises from about 5 to about 178 or about 179 contiguous amino acids of SEQ ID NOS:1, 2, 10 or 13. In particular, a human NGAL fragment comprises from about 5 to about 170 contiguous amino acids of SEQ ID NOS:1, 2, 10 or 13. In particular, a human NGAL fragment comprises at least about 5 contiguous amino acids of SEQ ID NOS:1, 2, 10 or 13, at least about 10 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13; at least about 15 contiguous amino acid residues of amino acids of SEQ ID NOS:1, 2, 10 or 13; at least about 20 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13; at least about 25 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 30 contiguous amino acid residues of amino acids of SEQ ID NOS:1, 2, 10 or 13, at least about 35 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 40 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 45 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 50 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 55 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 60 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 65 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 70 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 75 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 80 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 85 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 90 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 95 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 100 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 105 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 110 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 115 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 120 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 125 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 130 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 135 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 140 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 145 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 150 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 160 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13, at least about 165 contiguous amino acid residues of SEQ

ID NOS:1, 2, 10 or 13, at least about 170 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13 or at least about 175 contiguous amino acid residues of SEQ ID NOS:1, 2, 10 or 13.

**[0068]** Examples of human NGAL fragments contemplated by the present invention include, but are not limited to:

**[0069]** (a) a human NGAL fragment of at least about 7 contiguous amino acids which includes amino acid residues 112, 113, 114, 115, 116, 117 and 118 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue, and the signal peptide and any Met initiator residue(s) being numbered in the negative, as previously described herein);

**[0070]** (b) a human NGAL fragment of at least about 8 contiguous amino acids which includes amino acid residues 112, 113, 114, 115, 116, 117, 118 and 119 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue);

**[0071]** (c) a human NGAL fragment of at least about 36 contiguous amino acid which includes amino acid residues 112, 118 and 147 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue);

**[0072]** (d) a human NGAL fragment of at least about 95 contiguous amino acids which includes amino acid residues 15 and 109 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue);

**[0073]** (e) a human NGAL fragment of at least about 144 contiguous amino acids which includes amino acid residues 15, 109 and 158 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue);

**[0074]** (f) a human NGAL fragment of at least about 145 contiguous amino acids which includes amino acid residues 15, 109, 158 and 159 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue); or

**[0075]** (g) a human NGAL fragment of at least about 146 contiguous amino acids which includes amino acid residues 15, 109, 158, 159 and 160 of SEQ ID NOS:1, 2, 10 or 13 (with the numbering of SEQ ID NO:1 and 2 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue).

**[0076]** Optionally, such human NGAL fragments as described herein are encoded either in part or in the entirety by the corresponding sequences of SEQ ID NOS:3, 4 or 12. Along these lines, in one embodiment, the present invention provides an isolated, purified, or isolated and purified human NGAL polynucleotide comprising or consisting of the sequence of SEQ ID NOS:4 or 12.

#### i) NGAL Hybrid

**[0077]** As used herein, the term "NGAL hybrid" or "NGAL hybridoma" refers to a particular hybridoma clone or subclone (as specified) that produces an anti-NGAL antibody of interest. Generally, there may be some small variation in the

affinity of antibodies produced by a hybridoma clone as compared to those from a subclone of the same type, e.g., reflecting purity of the clone. By comparison, it is well established that all hybridoma subclones originating from the same clone and further, that produce the anti-NGAL antibody of interest produce antibodies of identical sequence and/or identical structure. NGAL hybrids are set forth in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

#### j) Specificity

**[0078]** "Specificity" for the amount of human NGAL as used herein is defined as the converse of cross-reactivity for dimer (i.e., specificity is equal to 1/cross-reactivity), such that a specificity of about 75% is equivalent to a cross-reactivity of about 25%, a specificity of about 90% is equivalent to a cross-reactivity of about 10%, etc. For purposes herein, an assay that is about 75% specific for NGAL monomer demonstrates about 25% cross-reactivity with NGAL dimer. An alternative way of stating this is that an assay with about 75% specificity for monomer is about 4 times as specific for NGAL monomer as compared to NGAL dimer.

#### k) Specific Binding

**[0079]** The term "specific binding" is defined herein as the preferential binding of one binding partner to another (e.g., two polypeptides, a polypeptide and nucleic acid molecule, or two nucleic acid molecules) at specific sites. The term "specifically binds" indicates that the binding preference (e.g., affinity) for the target molecule/sequence is at least 2-fold, more preferably at least 5-fold, and most preferably at least 10- or 20-fold over a non-specific target molecule (e.g. a random molecule lacking the specifically recognized site(s)).

#### l) Binding Partner

**[0080]** A "binding partner," as used herein, is a member of a binding pair, i.e., a pair of molecules wherein one of the molecules binds to the second molecule. Binding partners that bind specifically are termed "specific binding partners." In addition to the antigen and antibody binding partners commonly used in immunoassays, other specific binding partners can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding partners can include partner(s) that is/are analog(s) of the original specific binding partner, for example, an analyte-analog. Immunoreactive specific binding partners include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA methods.

#### m) Epitope

**[0081]** As used herein, the term "epitope", "epitopes" or "epitopes of interest" refer to a site(s) on any molecule that is recognized and is capable of binding to a complementary site(s) on its specific binding partner. The molecule and specific binding partner are part of a specific binding pair. For example, an epitope can be a polypeptide, protein, hapten, carbohydrate antigen (such as, but not limited to, glycolipids, glycoproteins or lipopolysaccharides) or polysaccharide and its specific binding partner, can be, but is not limited to, an antibody.

**[0082]** In particular, an epitope refers to a particular region (composed of one or more amino acids) of an antigen, namely a protein to which an antibody binds. More specifically, an antigenic epitope is the area on protein surface that interacts with the complementary area (paratope) on the surface of the antibody binding domains. The epitope thus participates in electrostatic interactions, hydrophobic interactions and hydrogen bonding with the antibody and also contains residues responsible for the correct geometry of the surface, its malleability and structural dynamics. There are also buried "second sphere" residues that carry a strong supporting role for the antigenic epitope.

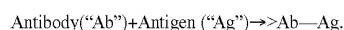
**[0083]** A particular type of epitope known as a "conformational epitope" or a "discontinuous epitope" is a type of epitope formed by residues that are sequentially discontinuous but close together in three-dimensional space.

n) Binding Constants (e.g.,  $K_D$ ,  $k_a$ , and  $k_d$ )

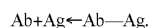
**[0084]** The terms "equilibrium dissociation constant" or " $K_D$ ", as used interchangeably herein, refer to the value obtained in a titration measurement at equilibrium, or by dividing the dissociation rate constant ( $k_{off}$ ) by the association rate constant ( $k_{on}$ ). The association rate constant, the dissociation rate constant and the equilibrium dissociation constant are used to represent the binding affinity of an antibody to an antigen.

**[0085]** The terms "relative affinity" or "relative  $K_R$ ", can be defined as the binding avidity of antibody to antigen revealed using the same test method to measure antibody/antigen  $K_D$  within a test population that includes antiserum test samples or uncloned hybrid test samples, thus providing relative affinity values rather than 'absolute' specificity data. (See, e.g., *Immunology*, 32:49 (1977) and *Essential Immunology*, Blackwell Scientific Publications, 7th edition, page 74 (1991)).

**[0086]** The term "association rate constant", " $k_{on}$ " or " $k_a$ " as used interchangeably herein, refers to the value indicating the binding rate of an antibody to its target antigen or the rate of complex formation between an antibody and antigen as shown by the equation below:



**[0087]** The term "dissociation rate constant", " $k_{off}$ " or " $k_d$ " as used interchangeably herein, refers to the value indicating the dissociation rate of an antibody from its target antigen or separation of Ab-Ag complex over time into free antibody and antigen as shown by the equation below:



**[0088]** Methods for determining association and dissociation rate constants are well known in the art. Using fluorescence-based techniques offers high sensitivity and the ability to examine samples in physiological buffers at equilibrium. Other experimental approaches and instruments such as a BIAcore® (biomolecular interaction analysis) assay can be used (e.g., instrument available from BIAcore International AB, a GE Healthcare company, Uppsala, Sweden). Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyn Instruments (Boise, Id.) can also be used.

o) Subject

**[0089]** As used herein, the terms "subject" and "patient" are used interchangeably irrespective of whether the subject has or is currently undergoing any form of treatment. As used herein, the terms "subject" and "subjects" refer to a mammal

including, a non-primate (for example, a cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, feline, canine, rat, and murine), a non-human primate (for example, a monkey, such as a cynomolgous monkey, chimpanzee, etc) and a human. Preferably, the subject is a human.

p) Test Sample

**[0090]** As used herein, the term "test sample" refers to a biological sample derived from serum, plasma, blood (including, but not limited to, whole blood), lymph, urine or other bodily fluids of a subject. The test sample can be prepared using routine techniques known to those skilled in the art. Preferably, the test sample is urine or blood.

q) Pretreatment Reagent (e.g., Lysis, Precipitation and/or Solubilization Reagent)

**[0091]** A pretreatment reagent used in a diagnostic assay as described herein is one that lyses any cells and/or solubilizes any analyte that are present in a test sample. Pretreatment is not necessary for all samples, as described further herein. Among other things, solubilizing the analyte (i.e., NGAL) entails release of the analyte from any endogenous binding proteins present the sample. A pretreatment reagent may be homogenous (not requiring a separation step) or heterogeneous (requiring a separation step). With use of a heterogeneous pretreatment reagent there is removal of any precipitated analyte binding proteins from the test sample prior to proceeding to the next step of the assay. The pretreatment reagent optionally can comprise: (a) one or more solvents and salt, (b) one or more solvents, salt and detergent, (c) detergent, (d) detergent and salt, or (e) any reagent or combination of reagents appropriate for cell lysis and/or solubilization of analyte. Also, proteases, either alone or in combination with any other pretreatment agents (e.g., solvents, detergents, salts, and the like) can be employed.

r) Solid Phase

**[0092]** A "solid phase," as used herein, refers to any material that is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize a capture agent. Alternatively, the solid phase can have affixed thereto a linking agent that has the ability to attract and immobilize the capture agent. The linking agent can, for example, include a charged substance that is oppositely charged with respect to the capture agent itself or to a charged substance conjugated to the capture agent. In general, the linking agent can be any binding partner (preferably specific) that is immobilized on (attached to) the solid phase and that has the ability to immobilize the capture agent through a binding reaction. The linking agent enables the indirect binding of the capture agent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase can, for example, be plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon, including, for example, a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

**[0093]** The terminology used herein is for the purpose of describing particular embodiments only and is not otherwise intended to be limiting.

## B. GLYCOSYLATED MAMMALIAN NGAL

**[0094]** Glycosylated mammalian NGAL employed in the context of the present invention (e.g., as a calibrator or con-

tol) is as described in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same). Generally, the present invention contemplates use of mammalian NGAL of any type (e.g., isolated, recombinant, mutant, wild-type, synthetic, semi-synthetic, and the like), especially mammalian NGAL that optionally is glycosylated, and particularly human NGAL as set forth herein. Such mammalian NGAL is employed, e.g., as immunogen for making antibodies, and/or in assessing binding of such antibodies.

**[0095]** In one embodiment, the present invention relates to isolated glycosylated mammalian NGAL. More specifically, the present invention relates to glycosylated mammalian NGAL that contains at least one oligosaccharide molecule or moiety and up to ten (10) oligosaccharide molecules or moieties. The glycosylated mammalian NGAL of the present invention includes, but is not limited to, glycosylated canine NGAL, glycosylated feline NGAL, glycosylated rat NGAL, glycosylated murine NGAL, glycosylated horse NGAL, glycosylated non-human primate NGAL and glycosylated human NGAL. Preferably, the glycosylated mammalian NGAL is human NGAL. Moreover, the glycosylated mammalian NGAL can be wild-type NGAL (namely, any wild-type mammalian NGAL, such as, but not limited to, wild-type canine NGAL, wild-type feline NGAL, wild-type rat NGAL, wild-type murine NGAL, wild-type horse NGAL, wild-type non-human primate NGAL or wild-type human NGAL). Preferably, the wild-type mammalian NGAL, is wild-type human NGAL having the amino acid sequence shown in SEQ ID NO:1 (including a signal peptide, and with the numbering of SEQ ID NO:1 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue) or SEQ ID NO:13 (not including a signal peptide). Alternatively, the glycosylated mammalian NGAL can be a glycosylated mutant mammalian NGAL that comprises an amino acid sequence comprising one or more amino acid substitutions, deletions or additions when compared to the corresponding amino acid sequence of the wild-type mammalian NGAL. For example, the glycosylated mammalian NGAL can be human NGAL wherein the amino acid sequence of the wild-type human NGAL (See, e.g., SEQ ID NOS:1 or 13) contains at least one amino acid substitution. Specifically, at least one amino acid substitution can be made at amino acid residue 87 of SEQ ID NOS:1 or 13. Specifically, the cysteine at amino acid 87 shown in SEQ ID NOS:1 or 13 can be replaced with a serine (See, e.g., SEQ ID NOS:2 and 10). Other substitutions for amino acids other than serine or cysteine can be made, e.g., glycine or alanine. Moreover, other amino acid substitutions, deletions or additions other than the single amino acid substitution at amino acid 87 of SEQ ID NOS:1 or 13 can be made by those skilled in the art using routine experimentation.

**[0096]** The mammalian NGAL employed herein (e.g., optionally glycosylated) can be made using recombinant DNA technology, by chemical synthesis or by a combination of chemical synthesis and recombinant DNA technology. Specifically, a polynucleotide sequence encoding mammalian NGAL may be constructed by isolating or synthesizing a polynucleotide sequence encoding the mammalian NGAL of interest. As mentioned above, the mammalian NGAL (e.g., optionally glycosylated) can be a wild-type mammalian NGAL or can be a mutant mammalian NGAL containing one or more amino acid substitutions, deletions or additions. Such amino acid substitutions, deletions or additions can be made

using routine techniques known in the art, such as by mutagenesis (for example, using site-directed mutagenesis in accordance with well known methods, e.g., as described in Nelson and Long, *Analytical Biochemistry* 180:147-151 (1989), random mutagenesis, or shuffling).

**[0097]** The polynucleotide sequence encoding the mammalian NGAL of interest may be prepared by chemical synthesis, such as by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired mammalian NGAL (wild-type or mutant), and by preferably selecting those codons that are favored in the host cell in which the recombinant mammalian NGAL will be produced. For example, several small oligonucleotides coding for portions of the desired mammalian NGAL may be synthesized and assembled by polymerase chain reaction (PCR), ligation or ligation chain reaction (LCR). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

**[0098]** Once assembled (such as by synthesis, site-directed mutagenesis or another method), the polynucleotide sequence encoding the mammalian NGAL of interest may be inserted into a recombinant vector and operably linked to any control sequences necessary for expression of thereof in the desired transformed host cell.

**[0099]** Although not all vectors and expression control sequences may function equally well to express a polynucleotide sequence of interest and not all hosts function equally well with the same expression system, it is believed that those skilled in the art will be able to easily make a selection among these vectors, expression control sequences, optimized codons, and hosts for use in the present invention without any undue experimentation. For example, in selecting a vector, the host must be considered because the vector must be able to replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors can also be considered. These include, but are not limited to, the relative strength of the sequence, its controllability, and its compatibility with the polynucleotide sequence encoding the mammalian NGAL, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, their codon usage, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, their ability (or lack thereof) to glycosylate the protein, and the ease of purification of the products coded for by the nucleotide sequence, etc.

**[0100]** The recombinant vector may be an autonomously replicating vector, namely, a vector existing as an extrachromosomal entity, the replication of which is independent of chromosomal replication (such as a plasmid). Alternatively, the vector can be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

**[0101]** The vector is preferably an expression vector, in which the polynucleotide sequence encoding the mammalian NGAL is operably linked to additional segments required for transcription of the polynucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts,

include, but are not limited to, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors include, pcDNA3.1 (+)\Hyg (Invitrogen Corp., Carlsbad, Calif.) and pCI-neo (Stratagene, La Jolla, Calif., USA). Examples of expression vectors for use in yeast cells include, but are not limited to, the 2 $\mu$ , plasmid and derivatives thereof, the POT1 vector (See, U.S. Pat. No. 4,931,373), the pJSO37 vector (described in Okkels, *Ann. New York Acad. Sci.*, 782:202-207, (1996)) and pPICZ A, B or C (Invitrogen Corp., Carlsbad, Calif.). Examples of expression vectors for use in insect cells include, but are not limited to, pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells" *Cell*, 45:685-698 (1986), pBluebac 4.5 and pMelbac (both of which are available from Invitrogen Corp., Carlsbad, Calif.). A preferred vector for use in the invention is pJV (available from Abbott Laboratories, Abbott Bioresearch Center, Worcester, Mass.).

**[0102]** Other vectors that can be used allow the polynucleotide sequence encoding the mammalian NGAL to be amplified in copy number. Such amplifiable vectors are well known in the art. These vectors include, but are not limited to, those vector that can be amplified by DHFR amplification (See, for example, Kaufman, U.S. Pat. No. 4,470,461, Kaufman et al., "Construction Of A Modular Dihydrofolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression" *Mol. Cell. Biol.*, 2:1304-1319 (1982)) and glutamine synthetase (GS) amplification (See, for example, U.S. Pat. No. 5,122,464 and EP Patent Application 0 338,841).

**[0103]** The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 $\mu$ , replication genes REP 1-3 and origin of replication.

**[0104]** The vector may also comprise a selectable marker, namely, a gene or polynucleotide, the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (See, P. R. Russell, *Gene*, 40: 125-130 (1985)), or one which confers resistance to a drug, such as, ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include, but are not limited to, amdS, pyrG, arcB, niaD and sC.

**[0105]** As used herein, the phrase "control sequences" refers to any components, which are necessary or advantageous for the expression of mammalian NGAL. Each control sequence may be native or foreign to the nucleic acid sequence encoding the mammalian NGAL. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence and transcription terminator. At a minimum, the control sequences include at least one promoter operably linked to the polynucleotide sequence encoding the mammalian NGAL.

**[0106]** As used herein, the phrase "operably linked" refers to the covalent joining of two or more polynucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, a polynucleotide sequence encoding a presequence or secre-

tory leader is operably linked to a polynucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the polynucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

**[0107]** A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, for example, the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (Ubc) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, *J Mol Biol.*, 196:947-50 (1987)).

**[0108]** In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the polynucleotide sequence encoding the mammalian NGAL. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

**[0109]** Examples of suitable control sequences for directing transcription in insect cells include, but are not limited to, the polyhedrin promoter, the P10 promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter and the SV40 polyadenylation sequence.

**[0110]** Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast a-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4-c promoter and the inducible GAL promoter.

**[0111]** Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger*  $\alpha$ -amylase, *A. niger* or *A. nidulus* glucoamylase, *A. nidulus* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator.

**[0112]** The polynucleotide sequence encoding the mammalian NGAL may or may not also include a polynucleotide sequence that encodes a signal peptide. The signal peptide is present when the mammalian NGAL is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide may be

homologous (for example, it may be that normally associated with the mammalian NGAL of interest) or heterologous (namely, originating from another source than the mammalian NGAL of interest) to the mammalian NGAL of interest or may be homologous or heterologous to the host cell, namely, be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, for example, derived from a bacterium, or eukaryotic, for example, derived from a mammalian, or insect, filamentous fungal or yeast cell.

**[0113]** The presence or absence of a signal peptide will, for example, depend on the expression host cell used for the production of the mammalian NGAL. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. For use in insect cells, the signal peptide may be derived from an insect gene (See, WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor, (See, U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen Corp., Carlsbad, Calif.), ecdysteroid UDP glucosyltransferase (egt) (Murphy et al., *Protein Expression and Purification* 4: 349-357 (1993), or human pancreatic lipase (hpl) (*Methods in Enzymology*, 284: 262-272 (1997)).

**[0114]** Specific examples of signal peptides for use in mammalian cells include murine Ig kappa light chain signal peptide (Coloma, M, *J. Imm. Methods*, 152:89-104 (1992)). For use in yeast cells suitable signal peptides include the  $\alpha$ -factor signal peptide from *S. cerevisiae* (See, U.S. Pat. No. 4,870,008), the signal peptide of mouse salivary amylase (See, O. Hagenbuchle et al., *Nature*, 289:643-646 (1981)), a modified carboxypeptidase signal peptide (See, L. A. Valls et al., *Cell*, 48:887-897 (1987)), the yeast BAR1 signal peptide (See, WO 87/02670), and the yeast aspartic protease 3 (YAP3) signal peptide (See, M. Egel-Mitani et al., *Yeast*, 6:127-137 (1990)).

**[0115]** Any suitable host may be used to produce the glycosylated mammalian NGAL of the present invention, including bacteria, fungi (including yeasts), plant, insect mammal or other appropriate animal cells or cell lines, as well as transgenic animals or plants. When a non-glycosylating organism such as *E. coli* is used, the expression in *E. coli* is preferably followed by suitable in vitro glycosylation in order to produce the glycosylated mammalian NGAL of the present invention.

**[0116]** Examples of bacterial host cells include, but are not limited to, gram positive bacteria such as strains of *Bacillus*, for example, *B. brevis* or *B. subtilis*, *Pseudomonas* or *Streptomyces*, or gram negative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (See, for example, Chang et al., *Molecular General Genetics*, 168: 111-115 (1979)), using competent cells (See, for example, Young et al., *Journal of Bacteriology*, 81:823-829 (1961)), or Dubnau et al., *Journal of Molecular Biology*, 56:209-221 (1971)), electroporation (See, for example, Shigekawa et al., *Biotechniques*, 6:742-751 (1988)), or conjugation (See, for example, Koehler et al., *Journal of Bacteriology*, 169:5771-5278 (1987)).

**[0117]** Examples of suitable filamentous fungal host cells include, but are not limited to, strains of *Aspergillus*, for example, *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* or

*Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall using techniques known to those skilled in the art. Suitable procedures for transformation of *Aspergillus* host cells are described in EP Patent Application 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., *Gene*, 78:147-156 (1989) and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., *Journal of Bacteriology*, 153:163 (1983); and Hinnen et al., *Proceedings of the National Academy of Sciences USA*, 75:1920 (1978).

**[0118]** Preferably, the mammalian NGAL of the present invention is glycosylated in vivo. When the mammalian NGAL is to be glycosylated in vivo, the host cell is selected from a group of host cells capable of generating the desired glycosylation of the mammalian NGAL. Thus, the host cell may be selected from a yeast cell, insect cell, or mammalian cell.

**[0119]** Examples of suitable yeast host cells include strains of *Saccharomyces*, for example, *S. cerevisiae*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. polymorpha* or *yarrowia*. Methods for transforming yeast cells with heterologous polynucleotides and producing heterologous polypeptides therefrom are disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit), and by Reeves et al., *FEMS Microbiology Letters*, 99:193-198 (1992), Manivasakam et al., *Nucleic Acids Research*, 21:4414-4415 (1993) and Ganeva et al., *FEMS Microbiology Letters*, 121:159-164 (1994).

**[0120]** Examples of suitable insect host cells include, but are not limited to, a *Lepidoptera* cell line, such as *Spodoptera frugiperda* (SF9 or Sf21) or *Trichoplusia ni* cells (High Five) (See, U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides are well known to those skilled in the art.

**[0121]** Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, Green Monkey cell lines (COS), mouse cells (for example, NS/O), Baby Hamster Kidney (BHK) cell lines, human cells (such as, human embryonic kidney cells (for example, HEK293 (ATCC Accession No. CRL-1573))) and plant cells in tissue culture. Preferably, the mammalian host cells are CHO cell lines and HEK293 cell lines. Another preferred host cell is the B3 cell line (e.g., Abbott Laboratories, Abbott Bioresearch Center, Worcester, Mass.), or another dihydrofolate reductase deficient (DHFR<sup>-</sup>) CHO cell line (e.g., available from Invitrogen Corp., Carlsbad, Calif.). In one aspect, the present invention relates to a CHO cell line which produces glycosylated human wild-type NGAL (namely, that which has the amino acid sequence of SEQ ID NOS:1 or 13), wherein the CHO cell line has been deposited with American Type Culture Collection (ATCC) on Nov. 21, 2006 and received ATCC Accession No. PTA-8020. Preferably, the wild-type human NGAL produced by the CHO cell line having ATCC Accession No. PTA-8020 has a molecular weight of about 25 kilodaltons (kDa). In another aspect, the present invention relates to a CHO cell line which produces glycosylated mutant human NGAL. Preferably, the glycosylated mutant human NGAL

comprises an amino acid substitution at the amino acid corresponding to amino acid 87 of the amino acid sequence of wild-type human NGAL (namely, SEQ ID NOS:1 or 13). More preferably, the amino acid substitution is the replacement of a cysteine with a serine (See, SEQ ID NOS:2 or 10). Most preferably, the CHO cell line is a CHO cell line that has been deposited with the ATCC on Jan. 23, 2007 and received ATCC Accession No. PTA-8168. The CHO cell line having ATCC Accession No. PTA-8168 produces a glycosylated mutant human NGAL comprising an amino acid sequence of SEQ ID NOS:2 or 10. In yet another aspect, the present invention relates to an isolated mutant glycosylated human NGAL comprising the amino acid sequence of SEQ ID NOS:2 or 10.

**[0122]** Methods for introducing exogenous polynucleotides into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamine™ 2000. These methods are well known in the art and are described, for example by Ausbel et al. (eds.) *Current Protocols in Molecular Biology* John Wiley & Sons, New York, USA (1996). The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in Jenkins, Ed., *Animal Cell Biotechnology, Methods and Protocols*, Human Press Inc. Totowa, N.J., USA (1999) and Harrison and Rae *General Techniques of Cell Culture*, Cambridge University Press (1997).

**[0123]** In the production methods, cells are cultivated in a nutrient medium suitable for production of the mammalian NGAL using methods known in the art. For example, cells are cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the glycosylated mammalian NGAL to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the glycosylated mammalian NGAL is secreted into the nutrient medium, the mammalian NGAL can be recovered directly from the medium. If the mammalian NGAL is not secreted, it can be recovered from cell lysates.

**[0124]** The resulting mammalian NGAL may be recovered by methods known in the art. For example, the mammalian NGAL may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

**[0125]** The mammalian NGAL may be purified by a variety of procedures known in the art including, but not limited to, chromatography (such as, but not limited to, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (such as, but not limited to, preparative isoelectric focusing), differential solubility (such as, but not limited to, ammonium sulfate precipitation), SDS-PAGE, or extraction (See, for example, J-C Janson and Lars Ryden, editors, *Protein Purification*, VCH Publishers, New York (1989)).

**[0126]** The glycosylated mammalian NGAL (wild-type and mutant) described herein can be used for a variety of different purposes and in a variety of different ways. Specifically, the glycosylated mammalian NGAL described herein can be used as one or more calibrators, one or more controls or as a combination of one or more calibrators or controls in an assay, preferably, an immunoassay, for detecting mammalian NGAL in a test sample. Glycosylated mammalian NGAL (wild-type and mutant) is part of the subject matter of U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding NGAL antigens, and calibrators or controls). Preferably, the glycosylated mammalian NGAL comprises the amino acid sequence of SEQ ID NOS:1 or 13. Alternatively, the glycosylated mammalian NGAL comprises the amino acid sequence of SEQ ID NOS:2 or 10.

**[0127]** For example, the glycosylated mammalian NGAL described herein can be used to improve the methods for detecting the presence of mammalian NGAL in a test sample. The exact steps of the method and the order of these steps for detecting mammalian NGAL in a test sample are not critical. Rather, the improvement in the method involves the use of the glycosylated mammalian NGAL described herein as one or more calibrators, one or more controls or as a combination of one or more calibrators and controls. For example, such a method might comprise the steps of:

**[0128]** (a) contacting a test sample suspected of containing mammalian NGAL with at least one antibody specific for the mammalian NGAL (such as, but not limited to, a capture antibody) for a time and under conditions that allow for the formation of a mammalian NGAL/antibody complex (such as a mammalian antibody/capture antibody complex); and

**[0129]** (b) detecting any mammalian NGAL/antibody complex formed (such as by adding a conjugate) as indicating the presence of the mammalian NGAL antigen,

**[0130]** wherein the method employs as at least one calibrator, at least one control, or as a combination of at least one calibrator or at least one control, at least one glycosylated mammalian NGAL described herein (such as a glycosylated mammalian NGAL having the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:10, or SEQ ID NO:13 or a combination of SEQ ID NOS:1, 2, 10 or 13 (namely, one used as a calibrator and one as a control, etc)).

**[0131]** One primary advantage for using mutant NGAL (e.g., as set forth in SEQ ID NOS:2 or 10), optionally glycosylated, is that calibrators made with wild-type NGAL could slowly form dimers over time. Because, as described in the examples herein traditional NGAL assays detect monomers better than dimers, this will be perceived as a loss of monomers (i.e., as an instability). The calibration curve will shift due to this, leading to decreased accuracy.

**[0132]** The mammalian NGAL (e.g., glycosylated mammalian NGAL) also optionally can be employed in kits for detecting the presence of NGAL in a sample as described herein.

**[0133]** Furthermore, the mammalian NGAL can be employed as immunogen to immunize animals for antibody production, e.g., where the animal can be a murine, rabbit, chicken, rat, sheep, goat, shark, camel, horse, feline, canine, non-human primate, human or other animal. In one embodiment, the immunogen comprises glycosylated mammalian NGAL, especially glycosylated human NGAL comprising the sequence of SEQ ID NO: 1, 2, 10 or 13. In another

embodiment, the mammalian NGAL is that of a canine, feline, rat, murine, horse, non-human primate, human, or other mammal.

### C. HUMAN NGAL ANTIBODIES

**[0134]** Antibodies directed against NGAL polypeptides, and methods of making such antibodies using NGAL polypeptides are described in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same). Such antibodies are further described herein in the context of their employ in the assays according to the invention. The present invention employs antibodies that specifically bind to wild-type human NGAL (namely, SEQ ID NOS:1 or 13) or human NGAL fragment. The antibodies also optionally bind to human NGAL wherein the amino acid sequence contains at least one amino acid substitution of the wild-type sequence (SEQ ID NOS:1 or 13) so as to comprise a mutant or non-native sequence (e.g., SEQ ID NOS:2 or 10).

**[0135]** In particular, in one aspect, the present invention employs isolated antibodies that bind to an epitope, e.g., a conformational epitope comprising the noncontiguous amino acid residues 112, 118 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13; with the numbering of SEQ ID NO:1 beginning at the Gln residue of the mature sequence immediately following the signal peptide and any Met initiator residue). In another aspect, the present invention employs isolated antibodies that bind to a conformational epitope comprising amino acid residues 112, 118 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13) and at least one (1) additional amino acid of human NGAL protein, wherein the additional amino acid is amino acid residue 117 or 119 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13). In yet another aspect, the present invention employs isolated antibodies that bind to a conformational epitope comprising amino acid residues 112, 117, 118, 119 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0136]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:5.

**[0137]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:5 and further wherein the antibody binds to: (1) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 117 of 119 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 112, 117, 118, 119 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0138]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable light domain region comprising an amino acid sequence of SEQ ID NO:6.

**[0139]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type

human NGAL, wherein the antibody has a variable light domain region comprising an amino acid sequence of SEQ ID NO:6 and further wherein the antibody binds to: (1) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 117 of 119 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 112, 117, 118, 119 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0140]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:5 and a variable light domain region comprising an amino acid sequence of SEQ ID NO:6.

**[0141]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:5 and a variable light domain region comprising an amino acid sequence of SEQ ID NO:6 and further wherein the antibody binds to: (1) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 117 of 119 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 112, 117, 118, 119 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0142]** In yet another aspect, the present invention relates to the use of antibodies as produced by a murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024, deposited on Nov. 21, 2006. The antibody produced by murine hybridoma cell line 1-2322-455 can bind to: (1) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 112, 118 and 147 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of human NGAL protein, wherein the additional amino acid is amino acid residue 117 of 119 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 112, 117, 118, 119 and 147 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13). Murine hybridoma cell line 1-2322-455 has a variable heavy domain comprising the amino acid sequence of SEQ ID NO:5 and a variable light domain comprising the amino acid sequence of SEQ ID NO:6.

**[0143]** In yet another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:7.

**[0144]** In yet another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:7 and further wherein the antibody binds to (1)

amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 15, 109, 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0145]** In yet another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable light domain region comprising an amino acid sequence of SEQ ID NO:8.

**[0146]** In yet another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable light domain region comprising an amino acid sequence of SEQ ID NO:8 and further wherein the antibody binds to (1) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 15, 109, 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0147]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:7 and a variable light domain region comprising an amino acid sequence of SEQ ID NO:8.

**[0148]** In another aspect, the present invention relates to the use of an isolated antibody that specifically binds to wild-type human NGAL, wherein the antibody has a variable heavy domain region comprising an amino acid sequence of SEQ ID NO:7 and a variable light domain region comprising an amino acid sequence of SEQ ID NO:8 and further wherein the antibody binds to: (1) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a conformational epitope comprising amino acid residues 15, 109, 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13).

**[0149]** In yet another aspect, the present invention relates to the use of an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026, deposited on Nov. 21, 2006. The antibody produced by murine hybridoma cell line 1-903-430 can bind to: (1) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13); (2) amino acid residues 15 and 109 of wild-type human NGAL protein (namely, SEQ ID NOS:1 or 13) and at least one additional amino acid of wild-type human NGAL protein, wherein the additional amino acid is amino acid residue 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13); or (3) to a

conformational epitope comprising amino acid residues 15, 109, 158, 159 or 160 of wild-type human NGAL (namely, SEQ ID NOS:1 or 13). Murine hybridoma cell line 1-903-430 has a variable heavy domain comprising the amino acid sequence of SEQ ID NO:7 and a variable light domain comprising the amino acid sequence of SEQ ID NO:8.

**[0150]** In still yet another embodiment, the present invention relates to the use of an isolated antibody that specifically binds to a human NGAL protein as set forth in SEQ ID NOS:1, 2, 10 or 13 (especially as set forth in SEQ ID NOS: 10 or 13),

**[0151]** wherein as a result of adding the antibody to the human NGAL protein (generally done in excess, particularly stoichiometric excess), the antibody causes as compared to when the antibody is not added,

**[0152]** (1) a perturbation of from about 0.05 ppm to about 1.0 ppm in a  $^1\text{H}$  resonance position, particularly from about 0.04 ppm to about 0.06 ppm, especially of about 0.05 ppm in a  $^1\text{H}$  resonance position,

**[0153]** (2) a perturbation of from about 0.3 ppm to about 3.0 ppm in a  $^{15}\text{N}$  resonance position, particularly of from about 0.1 ppm to about 2.0 ppm, especially of about 0.1 ppm, about 0.3 ppm, or about 0.6 ppm in a  $^{15}\text{N}$  resonance position, or

**[0154]** (3) from about a 2.5-fold to about a 20-fold decrease in resonance intensity, especially from about a 3-fold to about a 15-fold decrease, and particularly about a 4-fold to about a 10-fold decrease in resonance intensity,

**[0155]** in a TROSY proton-nitrogen correlation NMR spectra of at least three, four or five of the amide resonance positions for amino acids corresponding to residues of SEQ ID NOS:1 or 13, particularly from about two to six of the amide resonance positions for amino acids corresponding to residues of SEQ ID NOS:1, 2, 10 or 13 (especially of SEQ ID NOS: 10 or 13), selected from the group consisting of:

**[0156]** (a) for residue N116, a resonance position located at about  $^1\text{H}=9.47$  or about  $^{15}\text{N}=118.30$ ;

**[0157]** (b) for residue Q117, a resonance position located at about  $^1\text{H}=7.79$  or about  $^{15}\text{N}=117.67$ ;

**[0158]** (c) for residue H118, a resonance position located at about  $^1\text{H}=8.75$  or about  $^{15}\text{N}=116.43$ ;

**[0159]** (d) for residue T141, a resonance position located at about  $^1\text{H}=7.99$  or about  $^{15}\text{N}=109.06$ ;

**[0160]** (e) for residue K142, a resonance position located at about  $^1\text{H}=7.82$  or about  $^{15}\text{N}=114.25$ ;

**[0161]** (f) for residue E143, a resonance position located at about  $^1\text{H}=7.40$  or about  $^{15}\text{N}=114.00$ ; and

**[0162]** (g) for residue E150, a resonance position located at about  $^1\text{H}=8.70$  or about  $^{15}\text{N}=118.80$ . In other words, the shifts are in resonance positions that correspond to residues in the wild-type NGAL protein.

**[0163]** In still yet another embodiment, the present invention relates to the use of an isolated antibody that specifically binds to a human NGAL protein as set forth in SEQ ID NOS:1, 2, 10 or 13 (especially as set forth in SEQ ID NOS: 10 or 13),

**[0164]** wherein as a result of adding the antibody to the human NGAL protein (generally done in excess, particularly stoichiometric excess), the antibody causes as compared to when the antibody is not added,

**[0165]** (1) a perturbation of from about 0.05 ppm to about 1.0 ppm in a  $^1\text{H}$  resonance position, particularly from about 0.04 ppm to about 0.06 ppm, especially of about 0.05 ppm in a  $^1\text{H}$  resonance position,

**[0166]** (2) a perturbation of from about 0.3 ppm to about 3.0 ppm in a  $^{15}\text{N}$  resonance position, particularly of from about 0.1 ppm to about 2.0 ppm, especially of about 0.1 ppm, about 0.3 ppm, or about 0.6 ppm in a  $^{15}\text{N}$  resonance position, or

**[0167]** (3) from about a 2.5-fold to about a 20-fold decrease in resonance intensity, especially from about a 3-fold to about a 15-fold decrease, and particularly about a 4-fold to about a 10-fold decrease in resonance intensity,

**[0168]** in a TROSY proton-nitrogen correlation NMR spectra of at least three, four or five of the amide resonance positions for amino acids corresponding to residues of SEQ ID NOS:1 or 13, particularly from about two to six of the amide resonance positions for amino acids corresponding to residues of SEQ ID NOS:1, 2, 10 or 13 (especially of SEQ ID NOS: 10 or 13), selected from the group consisting of:

**[0169]** (a) for residue Y64, a resonance position located at about  $^1\text{H}=9.15$  or about  $^{15}\text{N}=113.30$ ;

**[0170]** (b) for residue V84, a resonance position located at about  $^1\text{H}=9.34$  or about  $^{15}\text{N}=121.50$ ;

**[0171]** (c) for residue G86, a resonance position located at about  $^1\text{H}=8.32$  or about  $^{15}\text{N}=111.60$ ;

**[0172]** (d) for residue T93, a resonance position located at about  $^1\text{H}=9.32$  or about  $^{15}\text{N}=112.80$ ;

**[0173]** (e) for residue L94, a resonance position located at about  $^1\text{H}=7.71$  or about  $^{15}\text{N}=122.72$ ;

**[0174]** (f) for residue G95, a resonance position located at about  $^1\text{H}=9.30$  or about  $^{15}\text{N}=113.70$ ; and

**[0175]** (g) for residue S99, a resonance position located at about  $^1\text{H}=8.18$  or about  $^{15}\text{N}=114.50$ .

#### D. METHODS OF MAKING AND USING NGAL ANTIBODIES

**[0176]** The antibodies employed in the immunoassays of the present invention can be made using a variety of different techniques known in the art. For example, polyclonal and monoclonal antibodies against wild-type human NGAL can be raised by immunizing a suitable subject (such as, but not limited to, a rabbit, goat, murine or other mammal) with an immunogenic preparation which contains a suitable immunogen. The immunogen that can be used for the immunization can include cells such as cells from immortalized cell lines NSO which is known to express human NGAL. In particular, antibodies as employed herein can be made as described in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

**[0177]** Alternatively, the immunogen can be the purified or isolated human wild-type NGAL protein itself (namely, SEQ ID NOS:1 or 13) or a human NGAL fragment thereof. For example, wild-type human NGAL (See, SEQ ID NOS:1 or 13) that has been isolated from a cell which produces the protein (such as NSO) using affinity chromatography, immunoprecipitation or other techniques which are well known in the art, can be used as an immunogen. Alternatively, immunogen can be prepared using chemical synthesis using routine techniques known in the art (such as, but not limited to, a synthesizer).

**[0178]** The antibodies raised in the subject can then be screened to determine if the antibodies bind to wild-type human NGAL or human NGAL fragment. Such antibodies can be further screened using the methods described herein (See, e.g., Example 5). For example, these antibodies can be assayed to determine if they bind to amino acid residues 112, 118 and 147 of wild-type human NGAL or amino acid resi-

dues 15 and 109 of wild-type human NGAL (See, SEQ ID NOS:1 or 13). Suitable methods to identify an antibody with the desired characteristics are described herein (See, Example, 5). Moreover, it is fully anticipated that results obtained with antibodies that bind to mutant NGAL (See, SEQ ID NOS:2 or 10), are fully translatable to binding of wild-type NGAL, and that antibodies will bind to comparable residues of wild-type human NGAL (See, SEQ ID NOS:1 or 13). Accordingly, for convenience, and unless there lacks a rational basis in a particular instance for not doing so, mutant NGAL can be employed to assess binding properties of antibodies.

**[0179]** The unit dose of immunogen (namely, the purified protein, tumor cell expressing the protein, or recombinantly expressed human NGAL protein) and the immunization regimen will depend upon the subject to be immunized, its immune status, and the body weight of the subject. To enhance an immune response in the subject, an immunogen can be administered with an adjuvant, such as Freund's complete or incomplete adjuvant.

**[0180]** Immunization of a subject with an immunogen as described above induces a polyclonal antibody response. The antibody titer in the immunized subject can be monitored over time by standard techniques such as an ELISA using an immobilized antigen, namely, human NGAL (SEQ ID NOS:1 or 13, or human NGAL fragment thereof) as described herein.

**[0181]** Other methods of raising antibodies against human NGAL (SEQ ID NOS:1 or 13, or a human NGAL fragment thereof) include using transgenic mice which express human immunoglobulin genes (See, for example, WO 91/00906, WO 91/10741 or WO 92/03918). Alternatively, human monoclonal antibodies can be produced by introducing an antigen into immune deficient mice that have been engrafted with human antibody-producing cells or tissues (for example, human bone marrow cells, peripheral blood lymphocytes (PBL), human fetal lymph node tissue, or hematopoietic stem cells). Such methods include raising antibodies in SCID-hu mice (See, for example, WO 93/05796, U.S. Pat. No. 5,411,749; or McCune et al., *Science*, 241:1632-1639 (1988)) or Rag-1/Rag-2 deficient mice. Human antibody-immune deficient mice are also commercially available. For example, Rag-2 deficient mice are available from Taconic Farms (Germantown, N.Y.).

**[0182]** Monoclonal antibodies can be generated by immunizing a subject with an immunogen. At the appropriate time after immunization, for example, when the antibody titers are at a sufficiently high level, antibody producing cells can be harvested from an immunized animal and used to prepare monoclonal antibodies using standard techniques. For example, the antibody producing cells can be fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique as originally developed by Kohler and Milstein, *Nature*, 256:495-497 (1975)), the human B cell hybridoma technique (Kozbar et al., *Immunology Today*, 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96 (1985)). The technology for producing monoclonal antibody hybridomas is well known to those skilled in the art.

**[0183]** Monoclonal antibodies can also be made by harvesting antibody producing cells, for example, splenocytes, from transgenic mice expressing human immunoglobulin genes

and which have been immunized with the human NGAL protein. The splenocytes can be immortalized through fusion with human myelomas or through transformation with Epstein-Barr virus (EBV). These hybridomas can be made using human B cell-or EBV-hybridoma techniques described in the art (See, for example, Boyle et al., European Patent Publication No. 0 614 984).

**[0184]** Hybridoma cells producing a monoclonal antibody which specifically binds to the wild-type human NGAL protein (SEQ ID NOS:1 or 13) or a human NGAL fragment thereof are detected by screening the hybridoma culture supernatants by, for example, screening to select antibodies that specifically bind to the immobilized human NGAL protein, or by testing the antibodies as described herein to determine if the antibodies have the desired characteristics, namely, the ability to bind to human NGAL at the amino acid residues described herein. After hybridoma cells are identified that produce antibodies of the desired specificity, the clones may be subcloned, e.g., by limiting dilution procedures, for example the procedure described by Wands et al. (*Gastroenterology* 80:225-232 (1981)), and grown by standard methods.

**[0185]** Hybridoma cells that produce monoclonal antibodies that test positive in the screening assays described herein can be cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium, to thereby produce whole antibodies. Tissue culture techniques and culture media suitable for hybridoma cells are generally described in the art (See, for example, R. H. Kennel, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, N.Y. (1980)). Conditioned hybridoma culture supernatant containing the antibody can then be collected. The monoclonal antibodies secreted by the subclones optionally can be isolated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

**[0186]** Monoclonal antibodies can be engineered by constructing a recombinant combinatorial immunoglobulin library and screening the library with the human NGAL protein. Kits for generating and screening phage display libraries are commercially available (See, for example, the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP Phage Display Kit, Catalog No. 240612). Likewise, yeast display vectors are known in the art and are commercially available (for example, pYD1 available from Invitrogen Corp., Carlsbad, Calif.). Briefly, the antibody library is screened to identify and isolate phages or yeast cells that express an antibody that specifically binds to the wild-type human NGAL protein (SEQ ID NOS:1 or 13). Preferably, the primary screening of the library involves screening with an immobilized wild-type human NGAL protein or a fragment thereof.

**[0187]** Following screening, the display phage or yeast is isolated and the polynucleotide encoding the selected antibody can be recovered from the display phage or yeast (for example, from the phage or yeast genome) and subcloned into other expression vectors (e.g., into *Saccharomyces cerevisiae* cells, for example EBY100 cells (Invitrogen Corporation, Carlsbad, Calif.)) by well known recombinant DNA techniques. The polynucleotide can be further manipulated (for

example, linked to nucleic acid encoding additional immunoglobulin domains, such as additional constant regions) and/or expressed in a host cell.

**[0188]** Alternatively, recombinant forms of antibodies, such as chimeric and humanized antibodies, can also be prepared to minimize the response by a human patient to the antibody. When antibodies produced in non-human subjects or derived from expression of non-human antibody genes are used therapeutically in humans, they are recognized to varying degrees as foreign, and an immune response may be generated in the patient. One approach to minimize or eliminate this immune reaction is to produce chimeric antibody derivatives, namely, antibody molecules that combine a non-human animal variable region and a human constant region. Such antibodies retain the epitope binding specificity of the original monoclonal antibody, but may be less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

**[0189]** Chimeric monoclonal antibodies can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the constant region of a non-human antibody molecule is substituted with a gene encoding a human constant region (See, for example, PCT Patent Publication PCT/US86/02269, European Patent Application 184, 187 or European Patent Application 171,496).

**[0190]** A chimeric antibody can be further "humanized" by replacing portions of the variable region not involved in antigen binding with equivalent portions from human variable regions. General reviews of "humanized" chimeric antibodies can be found in Morrison, S. L., *Science*, 229:1202-1207 (1985) and in Oi et al., *BioTechniques*, 4:214 (1986). Such methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of an immunoglobulin variable region from at least one of a heavy or light chain. The cDNA encoding the humanized chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable "humanized" antibodies can be alternatively produced by complementarity determining region (CDR) substitution (See, for example, U.S. Pat. No. 5,225,539; Jones et al., *Nature*, 321:552-525 (1986); Verhoeyan et al., *Science* 239:1.534 (1988); and Beidler et al., *J. Immunol*, 141:4053-4060 (1988)).

**[0191]** Epitope imprinting can also be used to produce a "human" antibody polypeptide dimer that retains the binding specificity of the antibodies (e.g., hamster antibodies) specific for the wild-type human NGAL protein (SEQ ID NOS:1 or 13) or human NGAL fragment thereof. Briefly, a gene encoding a non-human variable region (VH) with specific binding to an antigen and a human constant region (CH1), is expressed in *E. coli* and infected with a phage library of human V $\lambda$ .C $\lambda$  genes. Phage displaying antibody fragments are then screened for binding to the human NGAL protein. Selected human V $\lambda$  genes are recloned for expression of V $\lambda$ .C $\lambda$  chains and *E. coli* harboring these chains are infected with a phage library of human VHCH1 genes and the library is subject to rounds of screening with antigen coated tubes (See, WO 93/06213).

**[0192]** In another aspect, the present invention contemplates that the antibody is an antibody fragment. For example, the antibody fragment can include, but is not limited to, a Fab, a Fab', a Fab'-SH fragment, a di-sulfide linked Fv, a single chain Fv (scFv) and a F(ab')<sub>2</sub> fragment. Various techniques are known to those skilled in the art for the production of antibody fragments. For example, such fragments can be

derived via proteolytic digestion of intact antibodies (See, for example, Morimoto et al., *J. Biochem. Biophys. Methods*, 24:107-117 (1992) and Brennan et al., *Science*, 229:81 (1985)) or produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')<sub>2</sub> fragments (See, Carter et al., *Bio/Technology*, 10:163-167 (1992)). In another embodiment, the F(ab')<sub>2</sub> is formed using the leucine zipper GCN4 to promote assembly of the F(ab')<sub>2</sub> molecule. Alternatively, Fv, Fab or F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture. Single chain variable region fragments (scFv) are made by linking light and/or heavy chain variable regions by using a short linking peptide (See, Bird et al. *Science*, 242:423-426 (1998)). An example of a linking peptide is GPAKELTPLKEAKVS (SEQ ID NO:11). Linkers can in turn be modified for additional functions, such as attachment of drugs or attachment to solid supports. Examples of other linker sequences that can be used in the present invention can be found in Bird et al., *Science*, 242:423-426 (1998), Huston et al., *Proc. Natl. Acad. Sci. USA*, 85:5879-5883 (1988) and McCafferty et al., *Nature*, 348:552-554 (1990).

**[0193]** The single chain variants can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as *E. coli*. Polynucleotides encoding the scFv of interest can be made by routine manipulations such as ligation of polynucleotides. The resultant scFv can be isolated using standard protein purification techniques known in the art. Moreover, other forms of single chain antibodies, such as diabodies are also contemplated by the present invention. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (See, for example, Holliger, P., et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993); Poljak, R. J., et al., *Structure*, 2:1121-1123 (1994)).

**[0194]** Furthermore, in some aspects of the invention(s) as described herein (e.g., use as controls), it may be possible to employ commercially available anti-NGAL antibodies, or anti-NGAL antibodies or their methods for production described in the literature. These include but are not limited to: (1) anti-NGAL monoclonal antibodies (either HYB 211-01, HYB 211-02, or HYB 211-05, commercially available from AntibodyShop A/S, Gentofte, Denmark); (2) mouse anti-NGAL monoclonal antibody (e.g., Clone No. 697, Catalog No. HM2193B, HyCult Biotechnology, Uden, Netherlands); (3) rat anti-NGAL monoclonal antibody (e.g., Clone No. 220310, Catalog No. MAB1757, R&D Systems, Minneapolis, Minn.); (4) anti-NGAL antibodies contained in Quantikine® NGAL ELISA kit DLCN20 (R&D Systems, Minneapolis, Minn.), which purportedly detect the free NGAL form (i.e., form not complexed in a heterodimer) (U.S. Patent Application Publication No. 2007/0196876); (5) rabbit anti-human NGAL monoclonal antibodies produced in mouse hybridoma cells (EP 0 756 708 and U.S. Pat. No. 6,136,526); (6) purified monoclonal or polyclonal antibody against human NGAL (Kjeldsen et al., *J. Biol. Chem.*, 268:10425-

32 (1993); Kjeldsen et al., *J. Immunol. Methods*, 198(2): 155-64 (1996)); (7) polyclonal antibody against human NGAL (PCT International Application WO 2002/031507); and/or (8) discussing the use of solvent-exposed peptide loop areas of NGAL for making monoclonal antibody against human NGAL (U.S. Pat. No. 7,056,702 and U.S. Patent Application Publication US2004/0115728).

**[0195]** The antibodies of the present invention have a variety of uses. In one aspect, the antibodies of the present invention can be used as one or more immunodiagnostic reagents. For example, the antibodies of the present invention can be used as one or more immunodiagnostic reagents in one or more methods for detecting the presence of human NGAL antigen in a test sample. More specifically, the antibodies of the present invention can be used as one or more capture antibodies, one or more conjugate antibodies or as both one or more capture antibodies and one or more conjugate antibodies in immunoassays to detect the presence of human NGAL in a test sample.

#### E. SAMPLE COLLECTION AND PRETREATMENT

**[0196]** Methods well known in the art for collecting, handling and processing urine, blood, serum and plasma, and other body fluids, are used in the practice of the present invention.

**[0197]** The test sample may comprise further moieties in addition to the NGAL analyte of interest such as antibodies, antigens, haptens, hormones, drugs, enzymes, receptors, proteins, peptides, polypeptides, oligonucleotides or polynucleotides. For example, the sample may be a whole blood sample obtained from a subject. It may be necessary or desired that a test sample, particularly whole blood, be treated prior to immunoassay as described herein, e.g., with a pretreatment reagent. Even in cases where pretreatment is not necessary (e.g., most urine samples), pretreatment optionally may be done for mere convenience (e.g., as part of a regimen on a commercial platform). The pretreatment reagent can be a heterogeneous agent or a homogeneous agent.

**[0198]** With use of a heterogeneous pretreatment reagent according to the invention, the pretreatment reagent precipitates analyte binding protein (e.g., protein capable of binding NGAL) present in the sample. Such a pretreatment step comprises removing any analyte binding protein by separating from the precipitated analyte binding protein the supernatant of the mixture formed by addition of the pretreatment agent to sample. In such an assay, the supernatant of the mixture absent any binding protein is used in the assay, proceeding directly to the antibody capture step.

**[0199]** With use of a homogeneous pretreatment reagent there is no such separation step. The entire mixture of test sample and pretreatment reagent are contacted with the capture antibody in the antibody capture step. The pretreatment reagent employed for such an assay typically is diluted in the pretreated test sample mixture, either before the antibody capture step or during encounter with the antibody in the antibody capture step. Despite such dilution, a certain amount of the pretreatment reagent (for example, 5 M methanol and/or 0.6 M ethylene glycol) is still present (or remains) in the test sample mixture during antibody capture.

**[0200]** The pretreatment reagent can be any reagent appropriate for use with the immunoassay and kits of the invention. The pretreatment optionally comprises: (a) one or more solvents (e.g., methanol and ethylene glycol) and salt, (b) one or

more solvents, salt and detergent, (c) detergent, or (d) detergent and salt. Pretreatment reagents are known in the art, and such pretreatment can be employed, e.g., as used for assays on Abbott TDx, AxSYM®, and ARCHITECT® analyzers (Abbott Laboratories, Abbott Park, Ill.), as described in the literature (see, e.g., Yatscoff et al., Abbott TDx Monoclonal Antibody Assay Evaluated for Measuring Cyclosporine in Whole Blood, *Clin. Chem.*, 36:1969-1973 (1990) and Wallemacq et al., Evaluation of the New AxSYM Cyclosporine Assay: Comparison with TDx Monoclonal Whole Blood and EMIT Cyclosporine Assays, *Clin. Chem.* 45: 432-435 (1999)), and/or as commercially available. Additionally, pretreatment can be done as described in Abbott's U.S. Pat. No. 5,135,875, EP 0 471 293, U.S. Patent Application 60/878,017 filed Dec. 29, 2006; and U.S. patent application Ser. No. 11/490,624 filed Jun. 21, 2006 (incorporated by reference in its entirety for its teachings regarding pretreatment). Also, proteases, either alone or in combination with any other pretreatment agents (e.g., solvents, detergents, salts, and the like) can be employed.

#### F. NGAL IMMUNOASSAYS

**[0201]** Immunoassays can be conducted using any format known in the art, such as, but not limited to, a sandwich format. Specifically, in one aspect of the present invention, at least two antibodies are employed to separate and quantify human NGAL or human NGAL fragment in a test sample. More specifically, the at least two antibodies bind to certain epitopes of human NGAL or human NGAL fragment forming an immune complex which is referred to as a "sandwich". Generally, in the immunoassays one or more antibodies can be used to capture the human NGAL or human NGAL fragment in the test sample (these antibodies are frequently referred to as a "capture" antibody or "capture" antibodies) and one or more antibodies can be used to bind a detectable (namely, quantifiable) label to the sandwich (these antibodies are frequently referred to as the "detection antibody", "detection antibodies", a "conjugate" or "conjugates").

**[0202]** Excellent immunoassays, particularly, sandwich assays, can be performed using the antibodies of the present invention as the capture antibodies, detection antibodies or as capture and detection antibodies. For example, at least one of the antibodies of the present invention (such as antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-430 or a combination of an antibody produced by murine hybridoma cell line 1-2322-455 and an antibody produced by murine hybridoma cell line 1-903-430) can be used as a first capture antibody. Alternatively, in more than one capture antibody is being used, then the antibodies of the present invention can be used as a second or subsequent capture antibody. Alternatively, if one of the antibodies of the present invention is being used as a capture antibody, a different antibody (other than an antibody of the present invention) can be used as a second capture antibody. Alternatively, the antibodies of the present invention can be used only as detection antibodies and not as capture antibodies. Still in another alternative, the antibodies of the present invention can be used as both capture and detection antibodies. For example, an antibody produced by murine hybridoma cell line 1-2322-455 can be used as a capture antibody and an antibody produced by murine hybridoma cell line 1-903-422 can be used as a detection antibody. Alternatively, an antibody produced by murine hybridoma cell line 1-903-422 can be used as a

capture antibody and an antibody produced by hybridoma cell line 1-2322-455 can be used as a detection antibody.

**[0203]** The test sample being tested for (for example, suspected of containing) human NGAL or human NGAL fragment can be contacted with at least one capture antibody (or antibodies) and at least one detection antibody (which is either a second detection antibody or a third detection antibody) either simultaneously or sequentially and in any order. For example, the test sample can be first contacted with at least one capture antibody and then (sequentially) with at least one detection antibody. Alternatively, the test sample can be first contacted with at least one detection antibody and then (sequentially) with at least one capture antibody. In yet another alternative, the test sample can be contacted simultaneously with a capture antibody and a detection antibody.

**[0204]** In the sandwich assay format, a test sample suspected of containing human NGAL or human NGAL fragment is first brought into contact with an at least one first capture antibody under conditions which allow the formation of a first antibody/human NGAL complex. If more than one capture antibody is used, a first multiple capture antibody/human NGAL complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of human NGAL or human NGAL fragment expected in the test sample. For example, from about 5 µg/mL to about 1 mg/mL of antibody per mL of buffer (e.g., microparticle coating buffer) can be used.

**[0205]** Optionally, prior to contacting the test sample with the at least one capture antibody (for example, the first capture antibody), the at least one capture antibody can be bound to a solid support which facilitates the separation the first antibody/human NGAL complex from the test sample. Any solid support known in the art can be used, including, but not limited to, solid supports made out of polymeric materials in the forms of wells, tubes or beads. The antibody (or antibodies) can be bound to the solid support by adsorption, by covalent bonding using a chemical coupling agent or by other means known in the art, provided that such binding does not interfere with the ability of the antibody to bind human NGAL or human NGAL fragment. Alternatively, the antibody (or antibodies) can be bound with microparticles that have previously coated with streptavidin or biotin (for example, using Power-Bind™-SA-MP streptavidin coated microparticles, available from Seradyn, Indianapolis, Ind.). Alternatively, the antibody (or antibodies) can be bound using microparticles that have been previously coated with anti-species specific monoclonal antibodies. Moreover, if necessary, the solid support can be derivatized to allow reactivity with various functional groups on the antibody. Such derivatization requires the use of certain coupling agents such as, but not limited to, maleic anhydride, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

**[0206]** After the test sample being tested for and/or suspected of containing human NGAL or a human NGAL fragment is brought into contact with the at least one capture antibody (for example, the first capture antibody), the mixture is incubated in order to allow for the formation of a first antibody (or multiple antibody)-human NGAL complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2° C. to about 45° C., and for a period from at least about one (1) minute to about eighteen (18) hours, preferably from about 1 to about 20 minutes, most preferably for about 18 minutes. The immu-

noassay described herein can be conducted in one step (meaning the test sample, at least one capture antibody and at least one detection antibody are all added sequentially or simultaneously to a reaction vessel) or in more than one step, such as two steps, three steps, etc.

**[0207]** After formation of the (first or multiple) capture antibody/human NGAL complex, the complex is then contacted with at least one detection antibody (under conditions which allow for the formation of a (first or multiple) capture antibody/human NGAL/second antibody detection complex). The at least one detection antibody can be the second, third, fourth, etc. antibodies used in the immunoassay. If the capture antibody/human NGAL complex is contacted with more than one detection antibody, then a (first or multiple) capture antibody/human NGAL/(multiple) detection antibody complex is formed. As with the capture antibody (e.g., the first capture antibody), when the at least second (and subsequent) detection antibody is brought into contact with the capture antibody/human NGAL complex, a period of incubation under conditions similar to those described above is required for the formation of the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex. Preferably, at least one detection antibody contains a detectable label. The detectable label can be bound to the at least one detection antibody (e.g., the second detection antibody) prior to, simultaneously with or after the formation of the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex. Any detectable label known in the art can be used. For example, the detectable label can be a radioactive label, such as,  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ , an enzymatic label, such as horseradish peroxidase, alkaline phosphatase, glucose 6-phosphate dehydrogenase, etc., a chemiluminescent label, such as, acridinium esters, luminal, isoluminol, thioesters, sulfonamides, phenanthridinium esters, etc. a fluorescence label, such as, fluorescein (5-fluorescein, 6-carboxyfluorescein, 3'-6-carboxyfluorescein, 5(6)-carboxyfluorescein, 6-hexachloro-fluorescein, 6-tetrachlorofluorescein, fluorescein isothiocyanate, etc.), rhodamine, phycobiliproteins, R-phycoerythrin, quantum dots (zinc sulfide-capped cadmium selenide), a thermometric label or an immuno-polymerase chain reaction label. An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden, *Introduction to Immunocytochemistry*, 2<sup>nd</sup> ed., Springer Verlag, N.Y. (1997) and in Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (1996), which is a combined handbook and catalogue published by Molecular Probes, Inc., Eugene, Oreg.

**[0208]** The detectable label can be bound to the antibodies either directly or through a coupling agent. An example of a coupling agent that can be used is EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride) that is commercially available from Sigma-Aldrich, St. Louis, Mo. Other coupling agents that can be used are known in the art. Methods for binding a detectable label to an antibody are known in the art. Additionally, many detectable labels can be purchased or synthesized that already contain end groups that facilitate the coupling of the detectable label to the antibody, such as, N10-(3-sulfopropyl)-N-(3-carboxypropyl)-acridinium-9-carboxamide, otherwise known as CPSP-Acridinium Ester or N10-(3-sulfopropyl)-N-(3-sulfopropyl)-acridinium-9-carboxamide, otherwise known as SPSP-Acridinium Ester.

**[0209]** The (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the label. For example, if the at least one capture antibody (e.g., the first capture antibody) is bound to a solid support, such as a well or a bead, separation can be accomplished by removing the fluid (of the test sample) from contact with the solid support. Alternatively, if the at least first capture antibody is bound to a solid support it can be simultaneously contacted with the human NGAL-containing sample and the at least one second detection antibody to form a first (multiple) antibody/human NGAL/second (multiple) antibody complex, followed by removal of the fluid (test sample) from contact with the solid support. If the at least one first capture antibody is not bound to a solid support, then the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex does not have to be removed from the test sample for quantification of the amount of the label.

**[0210]** After formation of the labeled capture antibody/human NGAL/detection antibody complex (e.g., the first capture antibody/human NGAL/second detection antibody complex), the amount of label in the complex is quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex is reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label is quantified using a scintillation counter. If the label is a fluorescent label, the label is quantified by stimulating the label with a light of one color (which is known as the "excitation wavelength") and detecting another color (which is known as the "emission wavelength") that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of human NGAL or human NGAL fragment in the test sample is determined by use of a standard curve that has been generated using serial dilutions of human NGAL or human NGAL fragment of known concentration. Other than using serial dilutions of human NGAL or human NGAL fragment, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art.

**[0211]** In another aspect, the antibodies of the present invention can be used to determine the amount of human NGAL monomer in a test sample that is suspected of containing both human NGAL monomer and human NGAL dimer (such as human NGAL homodimer or human NGAL heterodimer). More specifically, the antibodies of the present invention can be used in one or more immunoassays to determine the amount of human NGAL monomer contained in a test sample with at least about seventy-five percent (75%) specificity.

**[0212]** More specifically, the antibodies of the present invention can be used as capture and detection antibodies to identify the amount of human NGAL monomer contained in a test sample. For example, if a first capture antibody comprising one or more antibodies such as an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422 is selected for use in an immunoassay, then the same or a different antibody can be selected for use as a second capture

antibody in addition to the first capture antibody. However, it is preferred that the second capture antibody bind to a different epitope than the epitope bound by the first capture antibody. Alternatively, the second capture antibody can be an antibody other than an antibody of the present invention. Alternatively, if a second capture antibody is not used, then the second antibody, namely, the detection antibody, can be one or more of antibodies of the present invention, such as, for example, antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422. As with the second capture antibody above, it is preferred that the detection antibody bind to a different epitope than the epitope bound by the first capture antibody, and if present, the second capture antibody.

**[0213]** Immunoassays performed as described herein that employ the antibodies of the present invention as at least one capture antibody or as at least one detection antibody are capable of determining the amount of human NGAL monomer contained in a test sample with at least about 75% specificity. Preferably, the immunoassay is capable of determining the amount of human NGAL monomer in a test sample with at least about 85% specificity. Most preferably, immunoassay is capable of determining the amount of human NGAL monomer in a test sample with at least about 90% specificity. Even more preferably, immunoassay is capable of determining the amount of human NGAL monomer in a test sample with at least about 95% specificity. Even most preferably, immunoassay is capable of determining the amount of human NGAL monomer in a test sample with at least about 98% specificity.

**[0214]** In particular, the methods and immunoassays as described herein optimally have from about 75% specificity up to about 98% specificity for monomer (e.g., are at least about four times, e.g., from about 3 to about 5 times, or at least about 3, 4, 4.5, or 5 times, as specific for NGAL monomer as compared to NGAL dimer), especially throughout the calibration range of the assay (e.g., from about 1 ng/mL up to about 2000 ng/ml of NGAL, especially urinary NGAL). Thus, the methods and immunoassays as described herein exhibit at least about 75% specificity from about 5 ng/mL up to about 2000 ng/ml of NGAL (e.g., including urinary NGAL), especially from about 100 ng/mL up to about 2000 ng/mL of NGAL (e.g., including urinary NGAL), particularly from about 150 ng/mL up to about 2000 ng/ml of NGAL (e.g., including urinary NGAL), including (a) from about 200 ng/mL up to about 2000 ng/ml of NGAL, (b) from about 250 ng/mL up to about 2000 ng/ml of NGAL, (c) from about 300 ng/mL up to about 2000 ng/ml of NGAL, (d) from about 350 ng/mL up to about 2000 ng/ml of NGAL, (e.g., including urinary NGAL), (e) from about 400 ng/mL up to about 2000 ng/ml of NGAL, and (a) from about 500 ng/mL up to about 2000 ng/ml of NGAL (e.g., including urinary NGAL).

**[0215]** The test sample being tested to determine the amount of human NGAL monomer can be contacted with at least one capture antibody (or antibodies) and at least one detection antibody (which is either a second detection antibody or a third detection antibody) either simultaneously or sequentially and in any order. For example, the test sample can be first contacted with at least one capture antibody and then (sequentially) with at least one detection antibody. Alternatively, the test sample can be first contacted with at least one detection antibody and then (sequentially) with at least one

capture antibody. In yet another alternative, the test sample can be contacted simultaneously with a capture antibody and a detection antibody.

**[0216]** In the sandwich assay format, a test sample is first brought into contact with the at least one first capture antibody which is an antibody of the present invention (such as, for example, an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422) under conditions which allow the formation of a first antibody/human NGAL complex. If more than one capture antibody is used, a first multiple capture antibody/human NGAL complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of human NGAL or human NGAL fragment expected in the test sample. For example, from about 5  $\mu$ g/mL to about 1 mg/mL of antibody per mL of buffer (e.g., micro-particle coating buffer) can be used.

**[0217]** Optionally, prior to contacting the test sample with the at least one capture antibody (e.g., the first capture antibody), the at least one capture antibody can be bound to a solid support using the techniques as discussed previously herein. After the test sample being suspected of containing human NGAL monomer and human NGAL dimer is brought into contact with the at least one capture antibody (e.g., the first capture antibody), the mixture is incubated in order to allow for the formation of a first antibody (or multiple antibody)/human NGAL complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2° C. to about 45° C., and for a period from at least about one (1) minute to about eighteen (18) hours, preferably from about 1 to about 20 minutes, most preferably for about 18 minutes. The immunoassay described herein can be conducted in one step (meaning the test sample, at least one capture antibody and at least one detection antibody are all added sequentially or simultaneously to a reaction vessel) or in more than one step, such as two steps, three steps, etc.

**[0218]** After formation of the (first or multiple) capture antibody/human NGAL complex, the complex is then contacted with at least one detection antibody (under conditions which allow for the formation of a (first or multiple) capture antibody/human NGAL/second antibody detection complex). The at least one detection antibody can be the second, third, fourth, etc. antibodies used in the immunoassay. If the capture antibody/human NGAL complex is contacted with more than one detection antibody, then a (first or multiple) capture antibody/human NGAL/(multiple) detection antibody complex is formed. As with the capture antibody (e.g., the first capture antibody), when the at least second (and subsequent) detection antibody is brought into contact with the capture antibody/human NGAL complex, a period of incubation under conditions similar to those described above is required for the formation of the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex. Preferably, at least one detection antibody contains a detectable label. The detectable label can be any detectable label as discussed previously herein.

**[0219]** The immunoassay described herein for determining the amount of human NGAL monomer in a test sample can be performed in the absence of any reducing agents. Alternatively, one or more of any of the steps of the immunoassay can be performed in the presence of one or more reducing agents. In yet another alternative, the test sample can be treated (namely, pre-treated) with one or more reducing agents prior

to using the test sample in the immunoassay. Any reducing agent can be used in the immunoassay or to pre-treat the test sample. Examples of reducing agents that can be used include, but are not limited to, dithiothreitol, 2-mercaptoethanol, 2-mercaptoethylamine and Tris (2-carboxyethyl)phosphine. The amount of reducing agent that can be used in the immunoassay or can be used to pre-treat the test sample is from about 0.1 mM to about 500 mM, especially an amount of from about 0.1 mM to about 100 mM.

**[0220]** The (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the label. For example, if the at least one capture antibody (e.g., the first capture antibody) is bound to a solid support, such as a well or a bead, separation can be accomplished by removing the fluid (of the test sample) from contact with the solid support. Alternatively, if the at least first capture antibody is bound to a solid support it can be simultaneously contacted with the human NGAL-containing sample and the at least one second detection antibody to form a first (multiple) antibody/human NGAL/second (multiple) antibody complex, followed by removal of the fluid (test sample) from contact with the solid support. If the at least one first capture antibody is not bound to a solid support, then the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex does not have to be removed from the test sample for quantification of the amount of the label.

**[0221]** After formation of the labeled capture antibody/human NGAL/detection antibody complex (e.g., the first capture antibody/human NGAL/second detection antibody complex), the amount of label in the complex is quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex is reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label is quantified using a scintillation counter. If the label is a fluorescent label, the label is quantified by stimulating the label with a light of one color (which is known as the "excitation wavelength") and detecting another color (which is known as the "emission wavelength") that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of human NGAL monomer in the test sample is determined by use of a standard curve that has been generated using serial dilutions of human NGAL monomer of known concentration. Other than using serial dilutions of human NGAL monomer, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art.

**[0222]** In yet another aspect, the antibodies of the present invention can be used in a method to determine the proportion of human NGAL monomer to human NGAL dimer contained in a test sample. The method for determining the proportion of human NGAL monomer to human NGAL dimer involves performing the steps of an immunoassay at least twice (or repeating certain steps of the immunoassay). More specifically, the antibodies of the present invention can be used as capture and detection antibodies to determine the proportion of human NGAL monomer to human NGAL dimer contained in a test sample. For example, if a first capture antibody

comprising one or more antibodies such as an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422 is selected for use in an immunoassay, then the same or a different antibody can be selected for use as a second capture antibody in addition to the first capture antibody. However, it is preferred that the second capture antibody bind to a different epitope than the epitope bound by the first capture antibody. Alternatively, the second capture antibody can be an antibody other than an antibody of the present invention. Alternatively, if a second capture antibody is not used, then the second antibody, namely, the detection antibody, can be one or more of antibodies of the present invention, such as, for example, antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422. As with the second capture antibody above, it is preferred that the detection antibody bind to a different epitope than the epitope bound by the first capture antibody, and if present, the second capture antibody.

**[0223]** Immunoassays performed as described herein not only employ the antibodies of the present invention as at least one capture antibody or as at least one detection antibody but also employ the use of one or more reducing agents at a specific point in the immunoassay. Any reducing agent can be used in the immunoassay. Examples of reducing agents that can be used include, but are not limited to, dithiothreitol, 2-mercaptoethanol, 2-mercaptoethylamine and Tris (2-carboxyethyl)phosphine. The amount of reducing agent that can be used in the immunoassay or can be used to pre-treat the test sample is from about 0.1 mM to about 500 mM, especially from about 0.1 mM to about 100 mM.

**[0224]** Specifically, the test sample being tested to determine proportion of human NGAL monomer to human NGAL dimer can be contacted with at least one capture antibody (or antibodies) and at least one detection antibody (which is either a second detection antibody or a third detection antibody) either simultaneously or sequentially and in any order. For example, the test sample can be first contacted with at least one capture antibody and then (sequentially) with at least one detection antibody. Alternatively, the test sample can be first contacted with at least one detection antibody and then (sequentially) with at least one capture antibody. In yet another alternative, the test sample can be contacted simultaneously with a capture antibody and a detection antibody.

**[0225]** In the sandwich assay format, a test sample is first brought into contact with the at least one first capture antibody which is an antibody of the present invention (such as, for example, an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422) under conditions which allow the formation of a first antibody/human NGAL complex. If more than one capture antibody is used, a first multiple capture antibody/human NGAL complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of human NGAL or human NGAL fragment expected in the test sample. For example, from about 5 µg/mL to about 1 mg/mL of antibody per mL of buffer (e.g., micro-particle coating buffer) can be used.

**[0226]** Optionally, prior to contacting the test sample with the at least one capture antibody (e.g., the first capture antibody), the at least one capture antibody can be bound to a solid support using the techniques as discussed previously herein. After the test sample being suspected of containing

human NGAL monomer and human NGAL dimer is brought into contact with the at least one capture antibody (e.g., the first capture antibody), the mixture is incubated in order to allow for the formation of a first (or multiple) antibody/human NGAL complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2° C. to about 45° C., and for a period from at least about one (1) minute to about eighteen (18) hours, preferably from about 1 to about 20 minutes, most preferably for about 18 minutes. The immunoassay described herein can be conducted in one step (meaning the test sample, at least one capture antibody and at least one detection antibody are all added sequentially or simultaneously to a reaction vessel) or in more than one step, such as two steps, three steps, etc.

**[0227]** After formation of the (first or multiple) capture antibody/human NGAL complex, the complex is then contacted with at least one detection antibody (under conditions which allow for the formation of a (first or multiple) capture antibody/human NGAL/second antibody detection complex). The at least one detection antibody can be the second, third, fourth, etc. antibodies used in the immunoassay. If the capture antibody/human NGAL complex is contacted with more than one detection antibody, then a (first or multiple) capture antibody/human NGAL/(multiple) detection antibody complex is formed. As with the capture antibody (e.g., the first capture antibody), when the at least second (and subsequent) detection antibody is brought into contact with the capture antibody/human NGAL complex, a period of incubation under conditions similar to those described above is required for the formation of the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex. Preferably, at least one detection antibody contains a detectable label. The detectable label can be any detectable label as discussed previously herein.

**[0228]** The (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the label. For example, if the at least one capture antibody (e.g., the first capture antibody) is bound to a solid support, such as a well or a bead, separation can be accomplished by removing the fluid (of the test sample) from contact with the solid support. Alternatively, if the at least first capture antibody is bound to a solid support it can be simultaneously contacted with the human NGAL-containing sample and the at least one second detection antibody to form a first (multiple) antibody/human NGAL/second (multiple) antibody complex, followed by removal of the fluid (test sample) from contact with the solid support. If the at least one first capture antibody is not bound to a solid support, then the (first or multiple) capture antibody/human NGAL/(second or multiple) detection antibody complex does not have to be removed from the test sample for quantification of the amount of the label.

**[0229]** After formation of the labeled capture antibody/human NGAL/detection antibody complex (e.g., the first capture antibody/human NGAL/second detection antibody complex), the amount of label in the complex is quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex is reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label is quantified using a scintillation counter. If the label is a fluorescent label, the label is quantified by stimulating the label with a light of one color (which is known as the “excitation wavelength”) and detecting another color (which is known as the “emission wavelength”) that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of human NGAL monomer in the test sample is determined by use of a standard curve that has been generated using serial dilutions of human NGAL monomer of known concentration. Other than using serial dilutions of human NGAL monomer, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art. Once the amount of human NGAL monomer has been determined, the ratio of human NGAL monomer to human NGAL dimer can be determined by comparing the amount of the (first or multiple) capture antibody/human NGAL complex determined when the immunoassay was performed without a reducing agent with the amount of the (first

tation wavelength”) and detecting another color (which is known as the “emission wavelength”) that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of human NGAL dimer and human monomer in the test sample is determined by use of a standard curve that has been generated using serial dilutions of human NGAL dimer and human NGAL monomer of known concentrations. Other than using serial dilutions of human NGAL dimer and human NGAL monomer, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art.

**[0230]** After the amount of human NGAL dimer and human monomer in the test sample is determined, the steps of the immunoassay are repeated. A new aliquot of the test sample is used in repeating the steps of the immunoassay (meaning that the aliquot used in repeating the steps of the immunoassay is different than the aliquot used to determine the amount of human NGAL dimer and human NGAL monomer). The steps of the immunoassay to be performed using the new aliquot of the test sample are identical to those described above, with the exception that one or more of the steps of the immunoassay are performed in the presence of at least one reducing agent. Alternatively, in lieu of performing one or more steps of the immunoassay in the presence of at least one reducing agent, the new aliquot of the test sample to be used repeating the steps of the immunoassay can be treated (namely, pretreated) with at least one reducing agent prior to performing the steps of the immunoassay. The use of the reducing agents in the immunoassay converts any dimer contained in the test sample to monomer. After formation of the labeled capture antibody/human NGAL/detection antibody complex, the amount of label in the complex is quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex is reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label is quantified using a scintillation counter. If the label is a fluorescent label, the label is quantified by stimulating the label with a light of one color (which is known as the “excitation wavelength”) and detecting another color (which is known as the “emission wavelength”) that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of human NGAL monomer in the test sample is determined by use of a standard curve that has been generated using serial dilutions of human NGAL monomer of known concentration. Other than using serial dilutions of human NGAL monomer, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art. Once the amount of human NGAL monomer has been determined, the ratio of human NGAL monomer to human NGAL dimer can be determined by comparing the amount of the (first or multiple) capture antibody/human NGAL complex determined when the immunoassay was performed without a reducing agent with the amount of the (first

or multiple) capture antibody/human NGAL complex determined when the immunoassay was repeated in with a reducing agent.

[0231] By way of example, the steps of the immunoassay can be performed as follows:

[0232] (a) contacting at least one capture first antibody that binds to human NGAL with a test sample suspected of containing human NGAL monomer and human NGAL dimer, to form a first antibody/human NGAL complex, wherein the at least one capture first antibody is an antibody selected from the group consisting of: an antibody produced by murine hybridoma cell line 1-2322-455, wherein the cell line has ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430, wherein the cell line has ATCC Accession No. PTA-8026;

[0233] (b) contacting the first antibody/human NGAL complex with a second antibody that binds to human NGAL and that has been conjugated to a detectable label to form a second antibody/human NGAL/first antibody complex, wherein the second antibody is an antibody selected from the group consisting of: an antibody produced by murine hybridoma cell line 1-2322-455, wherein the cell line has ATCC Accession No. PTA-8024 and an antibody produced by murine hybridoma cell line 1-903-430, wherein the cell line has ATCC Accession No. PTA-8026;

[0234] (c) determining the amount of the second antibody/human NGAL/first antibody complex formed in step (b), wherein steps (a) and (b) are performed in the absence of any reducing agents;

[0235] (d) repeating steps (a), (b) and (c) in the presence of or following treatment of the test sample with at least one reducing agent;

[0236] (e) determining the amount of the second antibody/human NGAL complex formed in the repeat of step (c); and

[0237] (f) determining the proportion of human NGAL monomer to human NGAL dimer in the test sample based on the amount of the second antibody/human NGAL/first antibody complex determined in step (c) and the amount of the second antibody/human NGAL/first antibody complex determined in step (e).

[0238] In another embodiment, the NGAL polypeptides also optimally can be employed in an improvement of an immunoassay for the detection of NGAL. This particular method as described herein can be employed in any NGAL immunoassay, with use of any antibodies for capture and/or detection. In one embodiment, the present invention thus provides, in an improvement of a method for detecting the presence of mammalian NGAL in a test sample, the method comprising:

[0239] (a) contacting a test sample suspected of containing mammalian NGAL with at least one antibody specific for said mammalian NGAL for a time and under conditions that allow the formation of a mammalian NGAL/antibody complex; and

[0240] (b) detecting any mammalian NGAL/antibody complex formed as indicating the presence of said mammalian NGAL;

[0241] wherein the improvement comprises employing as a calibrator or control a calibrator or control which is an NGAL polypeptide as described herein, particularly a

[0242] calibrator or control selected from the group consisting of: (a) glycosylated human NGAL comprising the sequence of SEQ ID NOS:2 or 10, and (b) glycosylated human NGAL comprising the sequence of SEQ ID NOS:1 or 13. In one variation of the improved NGAL assay, the cali-

brator or control is glycosylated human NGAL comprising the sequence of SEQ ID NOS:1 or 13.

[0243] Moreover, the anti-NGAL antibodies described for use herein also can be employed generally as an immunodiagnostic agent, e.g., in a method for detecting the presence of human NGAL antigen in a test sample. The method optionally comprises the steps of:

[0244] (1) contacting a test sample suspected of containing human NGAL with the immunodiagnostic reagent as described herein for a time and under conditions that allow formation of a human NGAL/antibody complex; and

[0245] (2) detecting any human NGAL/antibody complex formed as indicating the presence of the human NGAL antigen, wherein the immunodiagnostic reagent comprises one or more antibodies selected from the group consisting of:

[0246] (a) an antibody that specifically binds to a conformational epitope comprising amino acid residues 112, 118 and 147 of human NGAL protein as set forth in SEQ ID NOS:1, 2, 10 or 13;

[0247] (b) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5;

[0248] (c) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0249] (d) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0250] (e) an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024;

[0251] (f) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:7;

[0252] (g) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:8;

[0253] (h) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:7 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:8; and

[0254] (i) an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026.

[0255] The methods described herein (namely, the immunoassays and kits) can be used employed anytime any assessment of NGAL in any disease, disorder, condition, or evaluation of status is carried out. For instance, they can be used to evaluate the renal tubular cell injury status of a subject based on the determination of the level of NGAL present in the test sample. The subject to be evaluated can either currently have renal tubular cell injury or be at risk of developing renal tubular cell injury.

[0256] The methods described herein can be carried out on a subject after treatment of a subject for renal tubular cell injury or while the subject is currently experiencing renal tubular cell injury.

[0257] The methods described herein can be used to monitor the nephrotoxic side effects of drugs or other therapeutic agents in a subject.

[0258] The immunoassays also can be employed after an event experienced by a subject, such as after a surgical procedure (such as after cardiac surgery, coronary bypass surgery, cardiovascular surgery, vascular surgery or kidney transplantation), after the subject has experienced a diminished blood supply to the kidneys, if the subject has or is experiencing a medical condition selected from the group consisting of: impaired heart function, stroke, trauma, sepsis and dehydration, admittance of a subject to an intensive care unit, after administration to the subject of one or more pharmaceuticals, or after administration to the subject of one or more contrast agents.

[0259] The methods described herein also can be carried out or performed to assess chronic kidney disease.

[0260] It goes without saying that while certain embodiments herein are advantageous when employed to assess renal tubular cell injury status, the immunoassays and kits also optionally can be employed to assess NGAL in other diseases, e.g., cancer, sepsis, and any disease disorder or condition involving assessment of NGAL.

[0261] More specifically, in addition to assessment of renal disorders, diseases and injuries (see, e.g., U.S. Pat. App. Pub. Nos. 2008/0090304, 2008/0014644, 2008/0014604, 2007/0254370, and 2007/0037232), the assay and assay components as described herein optionally can also be employed in any other NGAL assay or in any other circumstance in which an assessment of NGAL levels or concentration might prove helpful: e.g., cancer-related assays (e.g., generally, or more specifically including but not limited to pancreatic cancer, breast cancer, ovarian/uterine cancer, leukemia, colon cancer, and brain cancer; see, e.g., U.S. Pat. App. Pub. No. 2007/0196876; see, also, U.S. Pat. Nos. 5,627,034 and 5,846,739); diagnosis of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, septic shock and multiple organ dysfunction syndrome (MODS) (see, e.g., U.S. Pat. App. Pub. Nos. 2008/0050832 and 2007/0092911; see, also, U.S. Pat. No. 6,136,526); hematology applications (e.g., estimation of cell type); assessment of preeclampsia, obesity (metabolic syndrome), insulin resistance, hyperglycemia, tissue remodeling (when complexed with MMP-9; see, e.g., U.S. Pat. App. Pub. No. 2007/0105166 and U.S. Pat. No. 7,153,660), autoimmune diseases (e.g., rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis), irritable bowel syndrome (see, e.g., U.S. Pat. App. Pub. Nos. 2008/0166719 and 2008/0085524), neurodegenerative disease, respiratory tract disease, inflammation, infection, periodontal disease (see, e.g., U.S. Pat. No. 5,866,432), and cardiovascular disease including venous thromboembolic disease (see, e.g., U.S. Pat. App. Pub. Nos. 2007/0269836), among others.

#### G. NGAL IMMUNOASSAY KITS

[0262] The present invention also contemplates kits for detecting the presence of mammalian NGAL antigen in a test sample in improved assays for monomer. Such kits can comprise one or more of the immunodiagnostic reagents (e.g., antibodies) described herein. More specifically, if the kit is a kit for performing an immunoassay, the kit optionally can contain (1) at least one capture antibody that specifically binds to mammalian NGAL; (2) at least one conjugate; and (3) one or more instructions for performing the immunoassay. The immunodiagnostic reagents of the present invention can

be included in such a test kit as a capture antibody, as a detection antibody or both as a capture antibody and a detection antibody. For example, an antibody produced by murine hybridoma cell line 1-2322-455 can be included in the kit as capture antibody and an antibody produced by murine hybridoma cell line 1-903-422 can be included in the kit as a detection antibody. Alternatively, an antibody produced by murine hybridoma cell line 1-903-422 can be included in the kit as a capture antibody and an antibody produced by hybridoma cell line 1-2322-455 can be included in the kit as a detection antibody. In still yet another alternative, an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422 can be included in the kit as a capture antibody and a different antibody included in the kit as a detection antibody. In still yet another alternative, an antibody produced by murine hybridoma cell line 1-2322-455 or an antibody produced by murine hybridoma cell line 1-903-422 can be included in the kit as a detection antibody and a different antibody included in the kit as a capture antibody. Optionally, the kit can also contain at least one calibrator or control. Any calibrator or control can be included in the kit. Preferably, however, the calibrator or control is mammalian NGAL, especially glycosylated human NGAL (e.g., wild-type or mutant) described previously herein.

[0263] Accordingly, the kits of the invention can comprise at least one calibrator, or at least one control, or a combination of at least one calibrator and at least one control, wherein the calibrator or control comprises a glycosylated mammalian NGAL of the present invention. Preferably, the at least one calibrator or at least one control is a glycosylated mammalian NGAL having the amino acid sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:13, and combinations of SEQ ID NOS:1, 2, 10 or 13. If the kit is a kit for performing an immunoassay, then the kit optionally further comprises: (1) at least one capture antibody that specifically binds to mammalian NGAL; (2) at least one conjugate; (3) one or more instructions for performing the immunoassay; or (4) or any combination of items (1)-(3).

[0264] Thus, the present invention further provides for diagnostic and quality control kits comprising one or more recombinant antibodies or mammalian NGAL of the invention. Optionally the assays, kits and kit components of the invention are optimized for use on commercial platforms (e.g., immunoassays on the Prism®, AxSYM®, ARCHITECT® and EIA (Bead) platforms of Abbott Laboratories, Abbott Park, Ill., as well as other commercial and/or in vitro diagnostic assays). Additionally, the assays, kits and kit components can be employed in other formats, for example, on electrochemical or other hand-held or point-of-care assay systems. The present invention is, for example, applicable to the commercial Abbott Point of Care (i-STAT®, Abbott Laboratories, Abbott Park, Ill.) electrochemical immunoassay system that performs sandwich immunoassays for several cardiac markers, including TnI, CKMB and BNP. Immunosensors and methods of operating them in single-use test devices are described, for example, in US Patent Applications 20030170881, 20040018577, 20050054078 and 20060160164 which are incorporated herein by reference. Additional background on the manufacture of electrochemical and other types of immunosensors is found in U.S. Pat. No. 5,063,081 which is also incorporated by reference for its teachings regarding same.

[0265] Optionally the kits include quality control reagents (e.g., sensitivity panels, calibrators, and positive controls). Preparation of quality control reagents is well known in the art, and is described, e.g., on a variety of immunodiagnostic product insert sheets. NGAL sensitivity panel members optionally can be prepared in varying amounts containing, e.g., known quantities of NGAL antigen or antibody ranging from “low” to “high”, e.g., by spiking known quantities of the NGAL antigen or antibodies according to the invention into an appropriate assay buffer (e.g., a phosphate buffer). These sensitivity panel members optionally are used to establish assay performance characteristics, and further optionally are useful indicators of the integrity of the immunoassay kit reagents, and the standardization of assays.

[0266] In another embodiment, the present invention provides for a quality control kit comprising one or more antigens and/or antibodies of the present invention for use as a sensitivity panel to evaluate assay performance characteristics and/or to quantitate and monitor the integrity of the antigen(s) used in the assay.

[0267] In still another embodiment, the mammalian NGAL (e.g., glycosylated mammalian NGAL) according to the invention can be employed as calibrators and/or controls. The antibodies provided in the kit can incorporate a detectable label, such as a fluorophore, radioactive moiety, enzyme, biotin/avidin label, chromophore, chemiluminescent label, or the like, or the kit may include reagents for labeling the antibodies or reagents for detecting the antibodies (e.g., detection antibodies) and/or for labeling the antigens or reagents for detecting the antigen. The antibodies, calibrators and/or controls can be provided in separate containers or pre-dispensed into an appropriate assay format, for example, into microtiter plates.

[0268] In yet another embodiment, the kit can comprise, either alone, with instructions, or with other aspects of the kit and kit components an immunodiagnostic agent that comprises one or more antibodies selected from the group consisting of:

[0269] (a) an antibody that specifically binds to a conformational epitope comprising amino acid residues 112, 118 and 147 of human NGAL protein as set forth in SEQ ID NOS:1, 2, 10 or 13;

[0270] (b) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5;

[0271] (c) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0272] (d) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:5 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:6;

[0273] (e) an antibody produced by murine hybridoma cell line 1-2322-455 having ATCC Accession No. PTA-8024;

[0274] (f) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:7;

[0275] (g) an isolated antibody that specifically bind to human NGAL, wherein the antibody has a variable light domain region comprising the amino acid sequence of SEQ ID NO:8;

[0276] (h) an isolated antibody that specifically binds to human NGAL, wherein the antibody has a variable heavy domain region comprising the amino acid sequence of SEQ ID NO:7 and a variable light domain region comprising the amino acid sequence of SEQ ID NO:8; and

[0277] (i) an antibody produced by murine hybridoma cell line 1-903-430 having ATCC Accession No. PTA-8026.

[0278] The kits can optionally include other reagents required to conduct a diagnostic assay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), may also be included in the kit. The kit may additionally include one or more other controls. One or more of the components of the kit may be lyophilized and the kit may further comprise reagents suitable for the reconstitution of the lyophilized components.

[0279] The various components of the kit optionally are provided in suitable containers. As indicated above, one or more of the containers may be a microtiter plate. The kit further can include containers for holding or storing a sample (e.g., a container or cartridge for a blood or urine sample). Where appropriate, the kit may also optionally contain reaction vessels, mixing vessels and other components that facilitate the preparation of reagents or the test sample. The kit may also include one or more instruments for assisting with obtaining a test sample, such as a syringe, pipette, forceps, measured spoon, or the like.

[0280] The kit further can optionally include instructions for use, which may be provided in paper form or in computer-readable form, such as a disc, CD, DVD or the like.

[0281] By way of example, and not of limitation, examples of the present invention shall now be given.

#### H. ADAPTATION OF METHOD AND ASSAY KIT

[0282] The kit (or components thereof), as well as the method of determining the concentration of NGAL antigen in a test sample by an assay as further described below, can be adapted for use in a variety of automated and semi-automated systems (including those wherein the solid phase comprises a microparticle), as described, e.g., in U.S. Pat. Nos. 5,089,424 and 5,006,309, and as commercially marketed, e.g., by Abbott Laboratories (Abbott Park, Ill.) as ARCHITECT®.

[0283] Some of the differences between an automated or semi-automated system as compared to a non-automated system (e.g., ELISA) include the substrate to which the first specific binding partner (e.g., NGAL capture antibody) is attached (which can impact sandwich formation and analyte reactivity), and the length and timing of the capture, detection and/or any optional wash steps. Whereas a non-automated format such as an ELISA may require a relatively longer incubation time with sample and capture reagent (e.g., about 2 hours) an automated or semi-automated format (e.g., ARCHITECT®, Abbott Laboratories) may have a relatively shorter incubation time (e.g., approximately 18 minutes for ARCHITECT®). Similarly, whereas a non-automated format such as an ELISA may incubate a detection antibody such as the conjugate reagent for a relatively longer incubation time (e.g., about 2 hours), an automated or semi-automated format

(e.g., ARCHITECT®) may have a relatively shorter incubation time (e.g., approximately 4 minutes for the ARCHITECT®).

**[0284]** Other platforms available from Abbott Laboratories include, but are not limited to, AxSYM®, IMx® (see, e.g., U.S. Pat. No. 5,294,404, which is hereby incorporated by reference in its entirety), PRISM®, EIA (bead), and Quantum™ II, as well as other platforms. Additionally, the assays, kits and kit components can be employed in other formats, for example, on electrochemical or other hand-held or point-of-care assay systems. The present disclosure is, for example, applicable to the commercial Abbott Point of Care (i-STAT®, Abbott Laboratories) electrochemical immunoassay system that performs sandwich immunoassays. Immunosensors and their methods of manufacture and operation in single-use test devices are described, for example in, U.S. Pat. No. 5,063,081, U.S. Pat. App. Pub. No. 2003/0170881, U.S. Pat. App. Pub. No. 2004/0018577, U.S. Pat. App. Pub. No. 2005/0054078, and U.S. Pat. App. Pub. No. 2006/0160164, which are incorporated in their entireties by reference for their teachings regarding same.

**[0285]** In particular, with regard to the adaptation of an NGAL assay to the I-STAT® system, the following configuration is preferred. A microfabricated silicon chip is manufactured with a pair of gold amperometric working electrodes and a silver-silver chloride reference electrode. On one of the working electrodes, polystyrene beads (0.2 mm diameter) with immobilized capture antibody are adhered to a polymer coating of patterned polyvinyl alcohol over the electrode. This chip is assembled into an I-STAT® cartridge with a fluidics format suitable for immunoassay. On a portion of the wall of the sample-holding chamber of the cartridge there is a layer comprising the second detection antibody labeled with alkaline phosphatase (or other label). Within the fluid pouch of the cartridge is an aqueous reagent that includes p-aminophenol phosphate.

**[0286]** In operation, a sample suspected of containing NGAL antigen is added to the holding chamber of the test cartridge and the cartridge is inserted into the I-STAT® reader. After the second antibody (detection antibody) has dissolved into the sample, a pump element within the cartridge forces the sample into a conduit containing the chip. Here it is oscillated to promote formation of the sandwich between NGAL antigen, NGAL capture antibody, and the labeled detection antibody. In the penultimate step of the assay, fluid is forced out of the pouch and into the conduit to wash the sample off the chip and into a waste chamber. In the final step of the assay, the alkaline phosphatase label reacts with p-aminophenol phosphate to cleave the phosphate group and permit the liberated p-aminophenol to be electrochemically oxidized at the working electrode. Based on the measured current, the reader is able to calculate the amount of NGAL antigen in the sample by means of an embedded algorithm and factory-determined calibration curve.

**[0287]** It further goes without saying that the methods and kits as described herein necessarily encompass other reagents and methods for carrying out the immunoassay. For instance, encompassed are various buffers such as are known in the art and/or which can be readily prepared or optimized to be employed, e.g., for washing, as a conjugate diluent, and/or as a calibrator diluent. An exemplary conjugate diluent is ARCHITECT® conjugate diluent employed in certain kits (Abbott Laboratories, Abbott Park, Ill.) and containing 2-(N-morpholino)ethanesulfonic acid (MES), a salt, a protein

blocker, an antimicrobial agent, and a detergent. An exemplary calibrator diluent is ARCHITECT® human calibrator diluent employed in certain kits (Abbott Laboratories, Abbott Park, Ill.), which comprises a buffer containing MES, other salt, a protein blocker, and an antimicrobial agent.

#### Example 1

##### Exemplary NGAL Assay (Native NGAL) Using Commercial Antibodies

**[0288]** This example illustrates the measurement of native NGAL (as described herein, meaning purified from in vivo, e.g., from a material such as blood) with an exemplary immunoassay that employs two commercially available antibodies.

**[0289]** Native NGAL was purchased from Diagnostics Development (Uppsala, Sweden). The presence of NGAL monomers and dimers was determined using reducing and non-reducing SDS-PAGE. The gels illustrated that the native NGAL as purchased was comprised mostly of dimer (data not shown). A small amount of monomer may have been present but this could not be quantitated from the gels.

**[0290]** For these studies, a 135 µL sample of the native NGAL was mixed with 15 µL of 10 mM dithiothreitol (DTT) for one hour at room temperature. For the control condition, 15 µL of water was substituted for 10 mM DTT. The assay was executed on an automated ARCHITECT® i2000 analyzer (Abbott Laboratories, Abbott Park, Ill.). The assay was carried out by:

**[0291]** 1. Mixing 10 µL of the test sample with 50 µL of microparticles coated with anti-NGAL antibody (HYB 211-01 from AntibodyShop A/S, Gentofte, Denmark).

**[0292]** 2. Incubating the reaction mixture for approximately 18 minutes at 33-38° C. The NGAL in the sample binds the anti-NGAL antibody on the microparticles.

**[0293]** 3. Washing the microparticles with a phosphate buffer.

**[0294]** 4. Adding 50 µL of acridinium-anti-NGAL antibody (HYB 211-02 from AntibodyShop A/S, Gentofte, Denmark) detector molecule to the reaction mixture.

**[0295]** 5. Incubating the reaction mixture for approximately 4 minutes at 33-38° C. The acridinium-anti-NGAL antibody molecule forms a sandwich with NGAL bound to the microparticle antibody.

**[0296]** 6. Washing the microparticles with a phosphate buffer.

**[0297]** 7. Adding Pre-trigger (acid solution) and Trigger (basic solution) to cause the captured acridinium-NGAL label to emit light, which is measured by the instrument as RLUs (Relative Light Units).

**[0298]** RLUs are the designation for the optical unit of measurement utilized on the ARCHITECT® systems. The ARCHITECT® optics system is essentially a photomultiplier tube (PMT) that performs photon counting on the light emitted by the chemiluminescent reaction. The amount of light generated by the chemiluminescent reaction is proportional to the amount of acridinium tracer present in the reaction mixture, and thereby allows quantitation of the patient sample analyte that is also proportional to the amount of acridinium remaining in the reaction mixture at the time the chemiluminescent reaction occurs.

**[0299]** The term "Relative Light Units" comes from the relation of the photon counting to a certain amount of acridinium. Each optics module is calibrated with a set of acridinium standards. When the chemiluminescent reaction

occurs, light is emitted and the photons are measured over a 3 second time period. The PMT converts the photons counted to digital signal, which is then sent to a circuit board for processing. The optics circuit board converts the digital signal from the PMT to an analog signal that is proportional to the photons counted, which is in turn proportional to the amount of acridinium present. This analog signal is then further processed to produce an RLU value. This relationship was established to produce a standard for calibration of the optics module, where the different acridinium standards have RLU values assigned to them. So, while the RLU unit itself is arbitrary, it is proportional (i.e., relative) to a certain amount of acridinium.

**[0300]** The assay response curve data obtained is shown in Table 1, below.

TABLE 1

NGAL (ng/mL)	RLUs
0	599
5	15330
50	150815
250	620011
600	1073295
1000	1321753

As can be seen from this data, and, as expected, in the exemplary NGAL assay using native NGAL, as the amount of NGAL increased, the signal detected in the assay increased.

### Example 2

#### Exemplary NGAL Assay Using Commercial Antibodies

**[0301]** This example compares the measurement of native NGAL monomer relative to native NGAL homodimer (dimer) with the exemplary immunoassay of Example 1.

**[0302]** The effect of the reduction of native NGAL by dithiothreitol ("DTT": 15  $\mu$ L of 10 mM DTT added to 135  $\mu$ L of sample, following by mixing and incubation for about an hour) on the measurement of NGAL also was explored, with results shown in Table 2 below.

TABLE 2

Native NGAL Control (non-reduced) (ng/mL)	Native NGAL (plus DTT - reduced) (ng/mL)
13.7	23.8

As can be seen from Table 2, following reduction of the native NGAL with DTT, the concentration of NGAL detected in the assay increased due to the conversion of NGAL dimer to NGAL monomer. This confirms that the immunoassay using commercial antibodies detects monomer preferentially to dimer. This further suggests that less NGAL is detected upon assay of non-reduced antigen samples with use of the immunoassay of Example 1, and implies that the true amount of NGAL in such samples potentially might be underestimated.

### Example 3

#### Exemplary NGAL Assay Using Recombinant Human NGAL

**[0303]** This example illustrates the preferential measurement of wild-type recombinant human NGAL monomer rela-

tive to wild-type recombinant human NGAL dimer with an exemplary immunoassay as described in Example 1 using the HYB 211-01 and HYB 211-02 antibodies.

**[0304]** All wild-type NGAL recombinant antigen (rAg) monomer or dimer, as well as any mutant C87S NGAL rAg clones, subclones, hybrids, and hybridomas (including names and numbering), vectors, vector constructs, and antibodies, not specifically described herein are described in their entirety in U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding NGAL antigens). For ease of reference, certain of these materials, in particular the illustrative depiction from U.S. Provisional Application Ser. No. 60/981,470 of FIG. 1 (which shows the human NGAL wild-type antigen sequence (SEQ ID NO:1); also is included herein.

**[0305]** Wild-type recombinant human NGAL expressed in CHO cells was purified by gel filtration chromatography as either monomer or as dimer. The presence of NGAL monomers and dimers was confirmed using reducing and non-reducing SDS-PAGE, following preparation of the samples by gel filtration chromatography. The SDS-PAGE confirmed that the dimer preparation was comprised mostly of dimer (data not shown). Also, calibrators were prepared with the monomer and dimer NGAL in 25 mM inorganic phosphate, 0.5 M NaCl, 1% Triton X-100, 0.05% BSA, 5  $\mu$ g/mL Sarafloxacin, 0.1%  $\text{NaN}_3$ , pH 7.5. A 200  $\mu$ L sample of each dimer calibrator was mixed with 5  $\mu$ L of 100 mM dithiothreitol (DTT) for two hours at room temperature. The assay was executed on an automated ARCHITECT® i2000 analyzer (Abbott Laboratories, Abbott Park, Ill.) as described in Example 1.

**[0306]** The response curve data are shown in Table 3 below and illustrate that (a) the immunoassay detects NGAL monomers preferentially to dimers and (b) conversion of dimers to monomers with reducing agent (e.g., DTT) makes the dimer calibrator assay response equivalent to the monomer calibrator response.

TABLE 3

NGAL Target Concentration (ng/mL)	RLUs		
	Monomer Calibrators	Dimer Calibrators	Dimer Calibrators plus DTT
0	748	728	872
5	12037	4047	11360
50	101307	31475	100508
250	396455	141698	378465
600	690890	282183	675772
1000	885013	389558	855181

**[0307]** Table 4, below, shows the same data following conversion to concentration units using the monomer calibrators as the reference standard. Following reduction of dimers to monomers, the concentration of NGAL increased by an average of 248% due to the conversion of dimers to monomers.

TABLE 4

NGAL Target Concentration (ng/mL)	Curve Fit Results (ng/mL)*			
	Monomer Calibrators	Dimer Calibrators	Dimer Calibrators plus DTT	% Increase due to DTT
0	0.0	0.0	0.1	—
5	5.1	1.4	4.7	229
50	49.5	14.1	49.0	247
250	251.1	71.8	235.8	229
600	597.9	161.3	574.2	256
1000	1001.5	245.2	924.3	277

\*Curve fit with monomer calibrators

## Example 4

## Immunoassay Performance Comparison Using Dimeric Vs. Monomeric NGAL Antigen

**[0308]** In order to determine the accuracy of various calibrators, monomer, dimer and DTT-treated (2.4 mM DTT) wild-type NGAL calibrators prepared as described in Example 3 were tested side-by-side on the ARCHITECT® NGAL assay using the HYB 211-01 and HYB 211-02 antibodies. Results obtained are presented in Table 5 (below).

TABLE 5

Calibrators	NGAL Concentration (ng/mL)	Monomer Calibrators (RLUs)	Dimer Calibrators (RLUs)	DTT-Treated Dimer Calibrators (RLUs)
A	0	747.5	728	872
B	5	12037	4046.5	11359.5
C	50	101307	31474.5	100507.5
D	250	396454.5	141698	378465
E	600	690890	282183	675772
F	1000	885013	389557.5	855180.5

**[0309]** As can be seen from Table 5, a primary advantage for using mutant NGAL is that calibrators made with wild-type NGAL could slowly form dimers over time. Because the assay “sees” monomers better than dimers, this will be perceived in the assay as a loss of monomers (i.e., an instability). The calibration curve will shift due to this phenomenon. Additionally, e.g., when employed for production of commercial product, dimers will form for the wild-type NGAL during the manufacturing process because of the high concentrations necessary to purify the NGAL. This would lead to a process wherein reducing agents would be necessary, thereby complicating the manufacturing process further and very likely leading to stability problems with the “reduced” antigen.

**[0310]** As can be seen from the above, there was near-quantitative recovery of the dimer signal to match the monomer signal upon adding DTT. This would indicate that the loss of signal in the dimer calibrators is actually due to the antibody pair not being able to recognize the dimer as well as the monomer.

## Example 5

## Exemplary NGAL Assay Using Subject NGAL Antibodies

**[0311]** This example illustrates the preferential measurement of recombinant human NGAL monomer relative to

NGAL dimer with an alternate format using an immunoassay comprised of antibodies produced by the 1-2322-455 and 1-903-430 cell lines, further as described in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same).

**[0312]** The recombinant human NGAL monomer and dimer calibrators described in Example 3 were used in this example. The assay was executed on an automated ARCHITECT® i2000 analyzer (Abbott Laboratories, Abbott Park, Ill.) as follows:

**[0313]** 1. Mixing 10  $\mu$ L of the sample (NGAL monomer or dimer calibrator) with 50  $\mu$ L of microparticles coated with anti-NGAL antibody produced by cell line 1-2322-455 and 20  $\mu$ L of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). TCEP is a disulfide bond reducing agent.

**[0314]** 2. Incubating the reaction mixture for approximately 18 minutes at 33-38° C. The NGAL in the sample binds the anti-NGAL antibody on the microparticles. TCEP reduces dimers to monomers.

**[0315]** 3. Adding 50  $\mu$ L of acridinium-anti-NGAL antibody produced by cell line 1-903-430 detector molecule to the reaction mixture.

**[0316]** 4. Incubating the reaction mixture for approximately 4 minutes at 33-38° C. The acridinium-anti-NGAL antibody molecule forms a sandwich with NGAL bound to the microparticle antibody.

**[0317]** 5. Washing the microparticles with a phosphate buffer.

**[0318]** 6. Adding Pre-trigger (acid solution) and Trigger (basic solution) to cause the captured acridinium-NGAL label to emit light, which is measured by the instrument.

**[0319]** The response curve data are shown in below in Table 6.

TABLE 6

NGAL Target Concentration (ng/mL)	RLUs			
	Monomer Calibrators	Monomer Calibrators plus TCEP	Dimer Calibrators	Dimer Calibrators plus TCEP
0	788	917	924	843
5	29336	29274	9117	14997
50	239507	241194	76836	131985
250	900324	928140	336240	535201
600	1505685	1528509	679832	988936
1000	1932662	1947234	992684	1371239

These results illustrate that the immunoassay detects NGAL monomers preferentially to dimers. It also shows that conversion of dimers to monomers can be accomplished with a reducing agent other than DTT, in this case, TCEP. Further, the reducing agent may be added during the assay incubation sequence as opposed to during a sample pretreatment step as described in Examples 1 and 2.

**[0320]** Table 7 shows the same data following conversion to concentration units using the monomer calibrators as the reference.

TABLE 7

NGAL Target Concentration (ng/mL)	Curve Fit Results (ng/mL)*			
	Monomer Calibrators	Monomer Calibrators plus TCEP	Dimer Calibrators	Dimer Calibrators plus TCEP
0	0.0	0.0	0.0	0.0
5	4.7	4.6	1.2	2.2
50	48.2	48.6	13.5	24.5
250	254.7	266.3	71.7	126.5
600	590.6	607.8	172.6	292.5
1000	1006.9	1025.6	294.2	497.4

\*Curve fit with monomer calibrators

### Example 6

#### Increased Specificity of ARCHITECT® Assay Compared to Commercial ELISA Kit

**[0321]** This example illustrates the preferential measurement of recombinant human NGAL monomer relative to NGAL dimer with an alternate format using an immunoassay comprised of antibodies produced by the 1-2322-455 and 1-903-430 cell lines, further as described in U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007 (incorporated by reference for its teachings regarding same) compared to the AntibodyShop NGAL Rapid ELISA Kit (AntibodyShop A/S, Gentofte, Denmark).

**[0322]** The recombinant human NGAL dimer calibrators described in Example 3 were used in this example. The dimer calibrators were used for these experiments at concentrations of 1500 ng/mL (“NGAL Dimer High”) and 150 ng/mL (“NGAL Dimer Low”).

**[0323]** The ARCHITECT® assay was executed on an automated ARCHITECT® i2000 analyzer (Abbott Laboratories, Abbott Park, Ill.) as follows:

**[0324]** 1. Mixing 10  $\mu$ L of the sample (NGAL dimer calibrator) with 90  $\mu$ L 0.5% saline.

**[0325]** 2. Incubating the reaction mixture for approximately 7 minutes at 33-38° C.

**[0326]** 3. Removing 25  $\mu$ L of this mixture and mixing with 50  $\mu$ L of microparticles coated with anti-NGAL antibody produced by cell line 1-2322-455 and 90  $\mu$ L ARCHITECT® line diluent (phosphate buffered saline with Brij detergent).

**[0327]** 4. Incubating the reaction mixture for approximately 18 minutes at 33-38° C. The NGAL in the sample binds the anti-NGAL antibody on the microparticles.

**[0328]** 5. Adding 50  $\mu$ L of acridinium-anti-NGAL antibody produced by cell line 1-903-430 detector molecule to the reaction mixture.

**[0329]** 6. Incubating the reaction mixture for approximately 4 minutes at 33-38° C. The acridinium-anti-NGAL antibody molecule forms a sandwich with NGAL bound to the microparticle antibody.

**[0330]** 7. Washing the microparticles with a phosphate buffer.

**[0331]** 8. Adding Pre-trigger (acid solution) and Trigger (basic solution) to cause the captured acridinium-NGAL label to emit light, which is measured by the instrument.

**[0332]** The AntibodyShop NGAL Rapid ELISA Kit was executed as described in the manufacturer’s instructions (Kit 037, Revision 2006-09-EN).

**[0333]** An exemplary response obtained with use of the NGAL dimer is shown below in Table 8.

TABLE 8

Sample	ELISA (ng/mL)	ARCHITECT (ng/mL)
NGAL Dimer High	477.6	308.6
NGAL Dimer Low	30.5	33.9

**[0334]** These results coupled with those, e.g., in Example 4 (showing improved specificity extending down to 5 ng/mL) confirm that the assay as described herein shows improved specificity throughout the calibration range of the assay (e.g., from about 1 ng/mL up to about 2000 ng/mL). The results furthermore confirm that the ARCHITECT® NGAL assay is more specific than the AntibodyShop NGAL Rapid ELISA Kit at higher dimer concentrations because the AntibodyShop NGAL Rapid ELISA Kit detects more dimer than the ARCHITECT® assay. It follows that at the higher concentrations of NGAL that are associated with acute kidney injury the ARCHITECT® NGAL assay is more specific for monomer than the AntibodyShop NGAL Rapid ELISA Kit. In other words, ARCHITECT® NGAL assay is more specific for monomer at a level of NGAL greater than about 100 ng/mL.

### Example 7

#### ATCC Deposit Information

**[0335]** The wild-type NGAL rAg CHO 662 cell line was deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, Va. 20110-2209 on Nov. 21, 2006 and received ATCC Accession No. PTA-8020.

**[0336]** The mutant NGAL rAg CHO C87S cell line (CHO cell clone #734, also known as “mutant C87S NGAL rAg CHO 734”) was deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, Va. 20110-2209 on Jan. 23, 2007 and received ATCC Accession No. PTA-8168.

**[0337]** Murine hybridoma cell lines 1-903-430 and 1-2322-455 were each deposited with the American Type Culture Collection (hereinafter referred to as “A.T.C.C”), University Blvd., Manassas, Va. 20110-2209, on Nov. 21, 2006. Cell line 1-903-430 was assigned ATCC Accession No. PTA-8026. Cell line 1-2322-455 was assigned ATCC Accession No. PTA-8024.

**[0338]** One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

**[0339]** All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. In particular, the following two U.S. Patent applications, co-filed with the present

disclosure, are incorporated by reference in their entireties: U.S. Provisional Application Ser. No. 60/981,471 filed Oct. 19, 2007; and U.S. Provisional Application Ser. No. 60/981,470 filed Oct. 19, 2007.

**[0340]** The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of” and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred

embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as encompassed by the appended claims. Moreover, it should be understood that where certain terms are defined under “Definitions” and are otherwise defined, described, or discussed elsewhere in the “Detailed Description,” all such definitions, descriptions, and discussions are intended to be attributed to such terms. There also is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof. Furthermore, while subheadings, e.g., “Definitions,” are used in the “Detailed Description,” such use is solely for ease of reference and is not intended to limit any disclosure made in one section to that section only; rather, any disclosure made under one subheading is intended to constitute a disclosure under each and every other subheading.

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SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 13

<210> SEQ ID NO 1

<211> LENGTH: 198

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<220> FEATURE:

<221> NAME/KEY: MISC\_FEATURE

<222> LOCATION: (1)..(20)

<223> OTHER INFORMATION: The first 20 amino acids are the signal peptide which would be labeled -1 to -20, with Gln (at position 21) being considered amino acid 1 of the NGAL peptide

<400> SEQUENCE: 1

Met Pro Leu Gly Leu Leu Trp Leu Gly Leu Ala Leu Leu Gly Ala Leu  
1                   5                   10                   15

His Ala Gln Ala Gln Asp Ser Thr Ser Asp Leu Ile Pro Ala Pro Pro  
20                   25                   30

Leu Ser Lys Val Pro Leu Gln Gln Asn Phe Gln Asp Asn Gln Phe Gln  
35                   40                   45

Gly Lys Trp Tyr Val Val Gly Leu Ala Gly Asn Ala Ile Leu Arg Glu  
50                   55                   60

Asp Lys Asp Pro Gln Lys Met Tyr Ala Thr Ile Tyr Glu Leu Lys Glu  
65                   70                   75                   80

Asp Lys Ser Tyr Asn Val Thr Ser Val Leu Phe Arg Lys Lys Lys Cys  
85                   90                   95

Asp Tyr Trp Ile Arg Thr Phe Val Pro Gly Cys Gln Pro Gly Glu Phe  
100                   105                   110

Thr Leu Gly Asn Ile Lys Ser Tyr Pro Gly Leu Thr Ser Tyr Leu Val  
115                   120                   125

Arg Val Val Ser Thr Asn Tyr Asn Gln His Ala Met Val Phe Phe Lys  
130                   135                   140

Lys Val Ser Gln Asn Arg Glu Tyr Phe Lys Ile Thr Leu Tyr Gly Arg  
145                   150                   155                   160

Thr Lys Glu Leu Thr Ser Glu Leu Lys Glu Asn Phe Ile Arg Phe Ser  
165                   170                   175

Lys Ser Leu Gly Leu Pro Glu Asn His Ile Val Phe Pro Val Pro Ile

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180                    185                    190

Asp Gln Cys Ile Asp Gly  
195

<210> SEQ ID NO 2  
<211> LENGTH: 198  
<212> TYPE: PRT  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized  
<220> FEATURE:  
<221> NAME/KEY: MISC\_FEATURE  
<222> LOCATION: (1)..(20)  
<223> OTHER INFORMATION: The first 20 amino acids are the signal  
peptide which would be labeled -1 to -20, with Gln (at  
position 21) being considered amino acid 1 of the NGAL peptide

<400> SEQUENCE: 2

Met Pro Leu Gly Leu Leu Trp Leu Gly Leu Ala Leu Leu Gly Ala Leu  
1                    5                    10                    15

His Ala Gln Ala Gln Asp Ser Thr Ser Asp Leu Ile Pro Ala Pro Pro  
20                    25                    30

Leu Ser Lys Val Pro Leu Gln Gln Asn Phe Gln Asp Asn Gln Phe Gln  
35                    40                    45

Gly Lys Trp Tyr Val Val Gly Leu Ala Gly Asn Ala Ile Leu Arg Glu  
50                    55                    60

Asp Lys Asp Pro Gln Lys Met Tyr Ala Thr Ile Tyr Glu Leu Lys Glu  
65                    70                    75                    80

Asp Lys Ser Tyr Asn Val Thr Ser Val Leu Phe Arg Lys Lys Lys Cys  
85                    90                    95

Asp Tyr Trp Ile Arg Thr Phe Val Pro Gly Ser Gln Pro Gly Glu Phe  
100                    105                    110

Thr Leu Gly Asn Ile Lys Ser Tyr Pro Gly Leu Thr Ser Tyr Leu Val  
115                    120                    125

Arg Val Val Ser Thr Asn Tyr Asn Gln His Ala Met Val Phe Phe Lys  
130                    135                    140

Lys Val Ser Gln Asn Arg Glu Tyr Phe Lys Ile Thr Leu Tyr Gly Arg  
145                    150                    155                    160

Thr Lys Glu Leu Thr Ser Glu Leu Lys Glu Asn Phe Ile Arg Phe Ser  
165                    170                    175

Lys Ser Leu Gly Leu Pro Glu Asn His Ile Val Phe Pro Val Pro Ile  
180                    185                    190

Asp Gln Cys Ile Asp Gly  
195

<210> SEQ ID NO 3  
<211> LENGTH: 612  
<212> TYPE: DNA  
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

atgccccctag gtctcctgtg gctgggacct gacctgttgg gggctctgca tgcccaggcc    60

caggactcca cctcagacct gatcccagcc ccacctctga gcaaggtecc tctgcagcag    120

aacttccagg acaaccaatt ccaggggaag tggtatgtgg taggctctggc agggaatgca    180

attctcagag aagacaaaga cccgcaaaag atgtatgcca ccatctatga gctgaaagaa    240

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gacaagagct acaatgtcac ctccgtcctg tttaggaaaa agaagtgtga ctactggatc 300
aggacttttg ttccagggtg ccagcccggc gagttcacgc tgggcaacat taagagttac 360
cctggattaa cgagttacct cgtcggagtg gtgagcacca actacaacca gcatgctatg 420
gtgttcttca agaaagtttc tcaaaacagg gagtacttca agatcacctc ctacggggaga 480
accaaggagc tgacttcgga actaaaggag aacttcatcc gcttctccaa atctctgggc 540
ctccctgaaa accacatcgt cttccctgtc ccaatcgacc agtgtatcga cggccatcat 600
caccatcacc at 612

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<210> SEQ ID NO 4
<211> LENGTH: 612
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically synthesized

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<400> SEQUENCE: 4

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atgccctag gtctcctgtg gctgggecta gccctgttgg gggctctgca tgcccaggcc 60
caggactcca cctcagacct gatcccagcc ccacctctga gcaaggccc tctgcagcag 120
aacttccagg acaaccaatt ccaggggaag tggatgtgg taggctggc agggaatgca 180
attctcagag aagacaaaaga cccgaaaag atgtatgcca ccactatga gctgaaagaa 240
gacaagagct acaatgtcac ctccgtcctg tttaggaaaa agaagtgtga ctactggatc 300
aggacttttg ttccagggtg ccagcccggc gagttcacgc tgggcaacat taagagttac 360
cctggattaa cgagttacct cgtcggagtg gtgagcacca actacaacca gcatgctatg 420
gtgttcttca agaaagtttc tcaaaacagg gagtacttca agatcacctc ctacggggaga 480
accaaggagc tgacttcgga actaaaggag aacttcatcc gcttctccaa atctctgggc 540
ctccctgaaa accacatcgt cttccctgtc ccaatcgacc agtgtatcga cggccatcat 600
caccatcacc at 612

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<210> SEQ ID NO 5
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically synthesized

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<400> SEQUENCE: 5

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10           15
Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asn Asn Tyr
20           25           30
Tyr Met Ser Trp Val Arg Gln Thr Pro Glu Arg Arg Leu Glu Trp Val
35           40           45
Ala Tyr Ile Ser Ser Ser Gly Gly Ser Thr Tyr Tyr Ser Asp Ser Val
50           55           60
Arg Gly Arg Phe Thr Ile Ser Arg Asp Thr Ala Arg Asn Thr Leu Tyr
65           70           75           80
Leu Gln Met Thr Ser Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys
85           90           95
Ala Arg His Phe Gly Asp Tyr Ser Tyr Phe Asp Tyr Trp Gly Gln Gly
100          105          110

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-continued

Thr Thr Leu Thr Val Ser Ser  
115

<210> SEQ ID NO 6  
<211> LENGTH: 108  
<212> TYPE: PRT  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized

<400> SEQUENCE: 6

Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly  
1 5 10 15  
Glu Thr Val Thr Ile Thr Cys Arg Ala Ser Glu Asn Phe Tyr Ser Tyr  
20 25 30  
Leu Ala Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val  
35 40 45  
Tyr Asn Ala Lys Thr Leu Ala Glu Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
Ser Gly Ser Gly Thr Gln Phe Ser Leu Lys Ile Asn Ser Leu Gln Pro  
65 70 75 80  
Glu Asp Phe Gly Thr Tyr Tyr Cys Gln His His Tyr Asp Ile Pro Leu  
85 90 95  
Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg  
100 105

<210> SEQ ID NO 7  
<211> LENGTH: 118  
<212> TYPE: PRT  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized

<400> SEQUENCE: 7

Lys Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu  
1 5 10 15  
Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr  
20 25 30  
Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met  
35 40 45  
Gly Trp Ile Asn Ile Asn Thr Gly Glu Pro Thr Tyr Ala Glu Glu Phe  
50 55 60  
Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Thr Thr Ala Phe  
65 70 75 80  
Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Leu Cys  
85 90 95  
Ala Arg Asp Ser Tyr Ser Gly Gly Phe Asp Tyr Trp Gly Gln Gly Thr  
100 105 110  
Ile Val Thr Val Ser Ser  
115

<210> SEQ ID NO 8  
<211> LENGTH: 114  
<212> TYPE: PRT  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized

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&lt;400&gt; SEQUENCE: 8

Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Val Ser Ala Gly  
 1            5                    10                    15

Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Ile Ser  
 20                    25                    30

Gly Asp Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln  
 35                    40                    45

Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Asp Ser Gly Val  
 50                    55                    60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Ala Asp Phe Thr Leu Thr  
 65                    70                    75                    80

Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn  
 85                    90                    95

Asp His Ser Phe Pro Pro Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu  
 100                    105                    110

Lys Arg

&lt;210&gt; SEQ ID NO 9

&lt;211&gt; LENGTH: 558

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Chemically synthesized

&lt;400&gt; SEQUENCE: 9

atgcaggact ctacttccga cctgattccg gctccgccgc tgtctaaagt gccgctgcag 60

cagaactttc aagacaacca gttccagggt aaatggtacg ttgtgggect ggetggtaac 120

gcgatcctgc gtgaagacaa agatccgcag aaaatgatg ctaccatcta cgaactgaaa 180

gaagacaaat cttataacgt gaccagcgtt ctgtttcgta aaaagaaatg tgactactgg 240

attcgacact tcgtgcccgg ctctcagccg ggcgagttca ctctgggtaa catcaaatct 300

taccggggtc tgactagcta cctgggtcgt gtggtttcta ctaactataa ccagcatgct 360

atgggtttct tcaagaaagt ttctcagaac cgtgaatact tcaagattac tetgtacggg 420

cgtaccaaag agctgacctc tgagctgaaa gaaaacttca tccgtttctc taaatctctg 480

ggcctgccgg agaaccatat cgtgtttccg gttccgatcg atcagtgcat cgacgggcat 540

catcaccatc accattga 558

&lt;210&gt; SEQ ID NO 10

&lt;211&gt; LENGTH: 178

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Chemically synthesized

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: MISC\_FEATURE

&lt;222&gt; LOCATION: (1)..(1)

&lt;223&gt; OTHER INFORMATION: Gln can be preceded by a Met residue (e.g., when synthetic and/or produced in prokaryotes)

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: MISC\_FEATURE

&lt;222&gt; LOCATION: (178)..(178)

&lt;223&gt; OTHER INFORMATION: Can, optionally, be succeeded at the C-terminus by one or more His residues, and especially, 6 His residues (HHHHHH).

&lt;400&gt; SEQUENCE: 10

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Gln Asp Ser Thr Ser Asp Leu Ile Pro Ala Pro Pro Leu Ser Lys Val  
 1 5 10 15  
 Pro Leu Gln Gln Asn Phe Gln Asp Asn Gln Phe Gln Gly Lys Trp Tyr  
 20 25 30  
 Val Val Gly Leu Ala Gly Asn Ala Ile Leu Arg Glu Asp Lys Asp Pro  
 35 40 45  
 Gln Lys Met Tyr Ala Thr Ile Tyr Glu Leu Lys Glu Asp Lys Ser Tyr  
 50 55 60  
 Asn Val Thr Ser Val Leu Phe Arg Lys Lys Lys Cys Asp Tyr Trp Ile  
 65 70 75 80  
 Arg Thr Phe Val Pro Gly Ser Gln Pro Gly Glu Phe Thr Leu Gly Asn  
 85 90 95  
 Ile Lys Ser Tyr Pro Gly Leu Thr Ser Tyr Leu Val Arg Val Val Ser  
 100 105 110  
 Thr Asn Tyr Asn Gln His Ala Met Val Phe Phe Lys Lys Val Ser Gln  
 115 120 125  
 Asn Arg Glu Tyr Phe Lys Ile Thr Leu Tyr Gly Arg Thr Lys Glu Leu  
 130 135 140  
 Thr Ser Glu Leu Lys Glu Asn Phe Ile Arg Phe Ser Lys Ser Leu Gly  
 145 150 155 160  
 Leu Pro Glu Asn His Ile Val Phe Pro Val Pro Ile Asp Gln Cys Ile  
 165 170 175  
 Asp Gly

<210> SEQ ID NO 11  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: scFV linking sequence

<400> SEQUENCE: 11

Gly Pro Ala Lys Glu Leu Thr Pro Leu Lys Glu Ala Lys Val Ser  
 1 5 10 15

<210> SEQ ID NO 12  
 <211> LENGTH: 552  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Chemically synthesized  
 <220> FEATURE:  
 <221> NAME/KEY: misc\_feature  
 <222> LOCATION: (1)..(1)  
 <223> OTHER INFORMATION: Optionally, can be preceded at the N-terminus  
 by an initiation codon encoding Met, namely, an ATG.

<400> SEQUENCE: 12

caggactcca cctcagacct gatcccagcc ccacctctga gcaaggtccc tctgcagcag 60  
 aacttccagg acaaccaatt ccaggggaag tggtatgtgg taggcctggc agggaatgca 120  
 attctcagag aagacaaga cccgcaaaa atgtatgcca ccatctatga gctgaaagaa 180  
 gacaagagct acaatgtcac ctccgtcctg tttaggaaaa agaagtgtga ctactggatc 240  
 aggacttttg ttccagggtc gcagcccggc gagttcacgc tgggcaacat taagagttac 300  
 cctggattaa cgagttacct cgctccgagtg gtgagcacca actacaacca gcatgctatg 360

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gtgttcttca agaagtttc tcaaacagc gagtacttca agatcacct ctacgggaga    420
accaaggagc tgacttcgga actaaaggag aacttcatcc gcttctccaa atctctgggc    480
ctccctgaaa accacatcgt cttccctgtc ccaatcgacc agtgtatcga cggccatcat    540
caccatcacc at                                                    552

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<210> SEQ ID NO 13
<211> LENGTH: 178
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically synthesized
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gln can be preceded by a Met residue (e.g.,
when synthetic and/or produced in prokaryotes.
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (178)..(178)
<223> OTHER INFORMATION: Can, optionally, be succeeded at the
C-terminus by one or more His residues, and especially, 6 His
residues (HHHHHH) .

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<400> SEQUENCE: 13

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Gln Asp Ser Thr Ser Asp Leu Ile Pro Ala Pro Pro Leu Ser Lys Val
1          5          10          15

Pro Leu Gln Gln Asn Phe Gln Asp Asn Gln Phe Gln Gly Lys Trp Tyr
20          25          30

Val Val Gly Leu Ala Gly Asn Ala Ile Leu Arg Glu Asp Lys Asp Pro
35          40          45

Gln Lys Met Tyr Ala Thr Ile Tyr Glu Leu Lys Glu Asp Lys Ser Tyr
50          55          60

Asn Val Thr Ser Val Leu Phe Arg Lys Lys Lys Cys Asp Tyr Trp Ile
65          70          75          80

Arg Thr Phe Val Pro Gly Cys Gln Pro Gly Glu Phe Thr Leu Gly Asn
85          90          95

Ile Lys Ser Tyr Pro Gly Leu Thr Ser Tyr Leu Val Arg Val Val Ser
100         105         110

Thr Asn Tyr Asn Gln His Ala Met Val Phe Phe Lys Lys Val Ser Gln
115         120         125

Asn Arg Glu Tyr Phe Lys Ile Thr Leu Tyr Gly Arg Thr Lys Glu Leu
130         135         140

Thr Ser Glu Leu Lys Glu Asn Phe Ile Arg Phe Ser Lys Ser Leu Gly
145         150         155         160

Leu Pro Glu Asn His Ile Val Phe Pro Val Pro Ile Asp Gln Cys Ile
165         170         175

Asp Gly

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**1.-31.** (canceled)

**32.** A method of diagnosing, monitoring or assessing the severity of disease or injury to an organ, tissue or cell type in a mammal by measuring the concentration of at least one individual molecular form of neutrophil gelatinase-associated lipocalin (NGAL) in a sample of bodily fluid from the mammal and comparing said concentration with the range of concentrations of said individual molecular form that occur in individuals not known to be affected by said disease or injury,

whereby a deviation of the concentration from said range determines the presence of disease or injury affecting said organ, tissue or cell type.

**33.** The method of claim **32** in which the individual molecular form of NGAL is free NGAL monomer.

**34.** The method of claim **32** in which the individual molecular form of NGAL is the NGAL homodimer or a higher oligomer.

35. The method of claim 32 in which the individual molecular form of NGAL is NGAL which has been glycosylated in a particular manner by its cells of origin, that particular manner being distinguishable from NGAL that has been glycosylated in another manner by different cells of origin.

36. A method of diagnosing, monitoring or assessing the severity of disease or injury to an organ, tissue or cell type in a mammal, said disease or injury being characterized by a certain pattern of concentrations of individual molecular forms of NGAL being present in a bodily fluid from said mammal, by measuring the concentrations of two or more individual molecular forms of NGAL in a sample of said bodily fluid and comparing the results with the pattern of said concentrations that occur in said disease or injury, whereby the presence of a particular pattern of concentrations indicates the presence of the corresponding type of disease or injury.

37. The method of claim 36 in which the concentration ratio between free NGAL monomer and NGAL homodimer is determined.

38. The method of claim 36 in which the concentration ratio between free NGAL monomer and the sum of NGAL homodimer plus higher oligomers of NGAL is determined.

39. The method of claim 36 in which the concentration ratio between molecular forms of NGAL that are free of matrix metalloproteinase 9 (MMP-9) and NGAL/MMP-9 complexes is determined.

40. A method according to claim 32 of diagnosing, monitoring or assessing the severity of a renal injury, disease or disorder which has led to acute renal failure or which signifies an immediate risk of developing acute renal failure in a mammal, said method comprising the steps of i) determining the concentration of free NGAL monomer in a sample of bodily fluid from the mammal and ii) comparing said concentration value with a cutoff value determined from the range of free NGAL monomer concentrations in other individuals of that mammalian species which show no evidence of renal injury, disease or disorder, so that a value greater than the cutoff value indicates the presence of said renal injury, disease or disorder.

41. A method according to claim 32 of diagnosing, monitoring or determining the risk of developing acute renal failure in a mammal, wherein said method discriminates between an individual which does not have acute renal failure and is not at immediate risk of developing acute renal failure, and an individual which may have acute renal failure or is at risk of developing acute renal failure, said method comprising the steps of i) determining the concentration of free NGAL monomer in a sample of bodily fluid from the mammal, and ii) comparing said concentration with a predetermined cutoff value chosen so that a concentration of free NGAL monomer below the cutoff value categorizes the mammal as not having and not being at immediate risk of developing acute renal failure.

42. The method of claim 40, comprising the further step of repeating steps i) and ii) of said claim one or more times.

43. The method of claim 41, comprising the further step of repeating steps i) and ii) of said claim one or more times.

44. The method of claim 40, comprising the further step of repeating steps i) and ii) of said claim within 24 hours, e.g. within 12 hours, such as within 6 hours, e.g. within 3 hours, such as within 1 hour, e.g. within 30 minutes or within 15 minutes.

45. The method of claim 41, comprising the further step of repeating steps i) and ii) of said claim within 24 hours, e.g. within 12 hours, such as within 6 hours, e.g. within 3 hours, such as within 1 hour, e.g. within 30 minutes or within 15 minutes.

46. The method of claim 40, comprising the further step of repeating steps i) and ii) of said claim after a treatment of acute renal failure has been initiated or completed.

47. The method of claim 41, comprising the further step of repeating steps i) and ii) of said claim after a treatment of acute renal failure has been initiated or completed.

48. The method of claim 40, wherein the renal injury or the risk of developing acute renal failure is due to ischemia.

49. The method of claim 41, wherein the renal injury or the risk of developing acute renal failure is due to ischemia.

50. The method of claim 40, wherein the renal injury or the risk of developing acute renal failure is due to a complication of an inflammatory, infective or neoplastic disease.

51. The method of claim 41, wherein the renal injury or the risk of developing acute renal failure is due to a complication of an inflammatory, infective or neoplastic disease.

52. The method of claim 40, wherein the renal injury or the risk of developing acute renal failure is due to critical illness of any cause requiring intensive care.

53. The method of claim 41, wherein the renal injury or the risk of developing acute renal failure is due to critical illness of any cause requiring intensive care.

54. The method of claim 40, wherein the renal injury or risk of developing acute renal failure is due to a surgical intervention.

55. The method of claim 41, wherein the renal injury or risk of developing acute renal failure is due to a surgical intervention.

56. The method of claim 40, wherein the renal injury or risk of developing acute renal failure is due to the administration of a nephrotoxic agent.

57. The method of claim 41, wherein the renal injury or risk of developing acute renal failure is due to the administration of a nephrotoxic agent.

58. The method of claim 32, wherein the injury is due to external physical or chemical causes.

59. The method of claim 36, wherein the injury is due to external physical or chemical causes.

60. The method of claim 40, wherein the injury is due to external physical or chemical causes.

61. The method of claim 32, wherein the injury is due to exposure to radiation.

62. The method of claim 36, wherein the injury is due to exposure to radiation.

63. The method of claim 32, wherein the bodily fluid is blood plasma.

64. The method of claim 36, wherein the bodily fluid is blood plasma.

65. The method of claim 40, wherein the bodily fluid is blood plasma.

66. The method of claim 41, wherein the bodily fluid is blood plasma.

67. The method of claim 32, wherein the bodily fluid is blood serum.

68. The method of claim 36, wherein the bodily fluid is blood serum.

69. The method of claim 40, wherein the bodily fluid is blood serum.

70. The method of claim 41, wherein the bodily fluid is blood serum.

71. The method of claim 32, wherein the bodily fluid is urine.

72. The method of claim 36, wherein the bodily fluid is urine.

73. The method of claim 40, wherein the bodily fluid is urine.

74. The method of claim 41, wherein the bodily fluid is urine.

75. The method of claim 32, wherein free monomer NGAL is measured by means of a molecule that binds specifically to free NGAL monomer and not to complexed forms of NGAL of the mammalian species to which the method is applied.

76. The method of claim 33, wherein free monomer NGAL is measured by means of a molecule that binds specifically to free NGAL monomer and not to complexed forms of NGAL of the mammalian species to which the method is applied.

77. The method of claim 36, wherein free monomer NGAL is measured by means of a molecule that binds specifically to free NGAL monomer and not to complexed forms of NGAL of the mammalian species to which the method is applied.

78. The method of claim 40, wherein free monomer NGAL is measured by means of a molecule that binds specifically to free NGAL monomer and not to complexed forms of NGAL of the mammalian species to which the method is applied.

79. The method of claim 41, wherein free monomer NGAL is measured by means of a molecule that binds specifically to free NGAL monomer and not to complexed forms of NGAL of the mammalian species to which the method is applied.

80. The method of claim 32, wherein NGAL homodimer or the sum of NGAL homodimer plus higher oligomers of NGAL is measured by a combination of binding molecules that are incapable of binding at one and the same time to free NGAL monomer.

81. The method of claim 34, wherein NGAL homodimer or the sum of NGAL homodimer plus higher oligomers of NGAL is measured by a combination of binding molecules that are incapable of binding at one and the same time to free NGAL monomer.

82. The method of claim 36, wherein NGAL homodimer or the sum of NGAL homodimer plus higher oligomers of

NGAL is measured by a combination of binding molecules that are incapable of binding at one and the same time to free NGAL monomer.

83. The method of claim 40, wherein NGAL homodimer or the sum of NGAL homodimer plus higher oligomers of NGAL is measured by a combination of binding molecules that are incapable of binding at one and the same time to free NGAL monomer.

84. The method of claim 41, wherein NGAL homodimer or the sum of NGAL homodimer plus higher oligomers of NGAL is measured by a combination of binding molecules that are incapable of binding at one and the same time to free NGAL monomer.

85. The method of claim 32, wherein the mammal is a human individual.

86. The method of claim 36, wherein the mammal is a human individual.

87. The method of claim 40, wherein the mammal is a human individual.

88. The method of claim 41, wherein the mammal is a human individual.

89. A kit or device for performing the method of claim 32, said kit or device comprising a solid support and binding molecules according to any of claims 75 through 84.

90. A kit or device for performing the method of claim 36, said kit or device comprising a solid support and binding molecules according to any of claims 75 through 84.

91. A kit or device for performing the method of claim 40, said kit or device comprising a solid support and binding molecules according to any of claims 75 through 84.

92. A kit or device for performing the method of claim 41, said kit or device comprising a solid support and binding molecules according to any of claims 75 through 84.

93. A method of selecting a binding molecule, including a monoclonal antibody, that is capable of binding specifically to NGAL monomer by selecting said molecule from a series of candidate binding molecules according to its ability to bind to a preparation of NGAL monomer and its inability to bind to NGAL homodimer.

\* \* \* \* \*

专利名称(译)	用于检测ngal的免疫测定和试剂盒		
公开(公告)号	<a href="#">US20100227775A1</a>	公开(公告)日	2010-09-09
申请号	US12/784548	申请日	2010-05-21
[标]申请(专利权)人(译)	雅培公司		
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当前申请(专利权)人(译)	亚培		
[标]发明人	BIRKENMEYER LARRY G DESAI SURESH M GRENIER FRANK C HAWKSWORTH DAVID J OLEJNICZAK EDWARD T RUAN QIAOQIAO SIEGEL ROBERT W TETIN SERGEY Y TIEMAN BRYAN C TU BAILIN TYNER JOAN D WORKMAN RYAN F ZIEMANN ROBERT N		
发明人	BIRKENMEYER, LARRY G. DESAI, SURESH M. GRENIER, FRANK C. HAWKSWORTH, DAVID J. OLEJNICZAK, EDWARD T. RUAN, QIAOQIAO SIEGEL, ROBERT W. TETIN, SERGEY Y. TIEMAN, BRYAN C. TU, BAILIN TYNER, JOAN D. WORKMAN, RYAN F. ZIEMANN, ROBERT N.		
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#### 摘要(译)

本发明涉及NGAL免疫测定和试剂盒，以及使用糖基化哺乳动物NGAL和在免疫测定和试剂盒中与哺乳动物NGAL结合的抗体的方法。其中，方法和试剂盒可用于测定测试样品中人NGAL单体的量，以及确定测试样品中包含的人NGAL单体与人NGAL二聚体的比例。

Signal Peptide

MPLGLLWLGL ALLGALHAQA QDSTSDLIPA PPLSKVPLQQ  
NFQDNQFQGK WYVVGLAGNA ILREDKDPQK MYATIYELKE  
DKSYNVTSVL FRKKKCDYWI RTFVPGCQPG EFTLGNIKSY  
PGLTSYLVRV VSTNYNQHAM VFFKKVSQNR EYFKITLYGR  
TKELTSELKE NFIRFSKSLG LPENHIVFPV PIDQCIDGHH

HHHH

6X His Tag