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(54) Title: SHORT TURNAROUND TIME INSULIN OR C-PEPTIDE ASSAY

(57) Abstract: The present invention relates to a real-time test system for use in methods to analyse samples on insulin or C-peptide. Particularly, the invention makes it possible to carry out the analysis close to a site wherein the test results are needed. The present invention is especially suitable in intra-operative and diabetes related (i.e. beta-cells transplantation) applications. The invention can be used for *in* and *ex vivo* applications.

SHORT TURNAROUND TIME INSULIN OR C-PEPTIDE ASSAY

The present invention is in the field of insulin-related or -associated diseases and discomforts. More in particular, it relates to a fast insulin assay with a short turnaround time. The invention makes it possible to carry out the assay or analysis close to a site wherein the test results are needed. The present invention is especially suitable in intra-operative applications. The invention can be used for *in* and *ex vivo* applications.

Insulin is produced by beta cells in pancreatic islets of Langerhans via a complex process of proteolytic conversion. The precursor pro-insulin is transported to the Golgi apparatus where it is packed into secretory granules. Maturation of the secretory granules is associated with conversion of pro-insulin to insulin and C-peptide by enzymatic cleavage. Secretion of insulin into the bloodstream is accompanied by the release of small amounts of pro-insulins.

There are quite a number of diseases and discomforts associated with and related to abnormal insulin excretions. The present invention is useful in this field.

For instance, with an incidence of 4 cases per million persons per year, insulinomas are the most common islet cell tumors. In 80% to 90% of these cases, organic hypoglycemia and hyperinsulinism are secondary to a sporadic, solitary benign insulin-secreting intrapancreatic tumor. Preoperatively, tumors with a size of about 1 cm and higher can be detected using a number of techniques. Among available techniques for pancreatic imaging, morphologic tools such as somatostatin receptor scintigraphy, endoscopic ultrasonography (USG), helical computed tomography (CT), or functional localization with intra-arterial stimulation (IAS) tests appear to be of particular value.

In Surgery, 128 (2000) 386-391, Berends *et al.* describe that the various known pre-operative imaging techniques to find insulinomas have a

success rate of about 60%. In addition, they describe that laparascopic detection by laparoscopic ultrasonography could identify insulinomas in 90% of the patients.

Hashimoto and Walsh describe in an article in Elsevier Science Inc., 189 (1999) 368-373 that preoperative localization of insulinomas is not necessary. On the basis of statistics, they conclude that the diagnosis of an insulinoma does not require extensive localization studies before operation. More in particular, they come to this conclusion because the combination of surgical exploration and intraoperative ultrasonography identified more than 90% of insulinomas. The resections and enucleations are retrospectively reviewed.

In US-A-4,104,029, an assay is described for determining immunologically and biochemically active compounds in for instance blood, offering the possibility to determine extremely low concentrations. The assay comprises mixing a sample to be tested with an antibody and with a chemiluminescent label, wherein the optionally present ligand partially reacts with the antibody and partially reacts with the label, during incubation. Subsequently, free and labelled ligand are separated and an activator is added. Using luminescence the respective amounts can be determined. Insulin is mentioned as ligand in this patent. However, one cannot use this method as a real-time system. The separation steps take for instance too much time; the method described cannot be carried out in e.g. half an hour.

EP-A-0 484 961 relates to a method for determining human C-peptide. Use can be made of a monoclonal antibody and labelled human C-peptide. After incubation the immunoreaction products formed are separated from labelled C-peptide. The method as described takes too much time to be a real-time method.

WO-A-98/59246 discloses a C-peptide specific assay using antibodies for the determination of the amounts of C-peptide. The antibodies used not only recognise proinsulin but also its intermediates like 31,32-proinsulin and

64,65-proinsulin. Example 1 points to a system requiring already an incubation time of 30 minutes, while Example 3 mentions a two-hour incubation step.

Intraoperatively, a careful pancreatic manual exploration completed with intraoperative ultrasonography (IOUS) leads to the detection of about 90% of these solitary insulinomas. Virtually all patients with such a solitary lesion are eventually cured after the first surgical exploration by enucleation of the tumor(s) when feasible or formal pancreatic resection when necessary. Conversely, surgical treatment of organic hypoglycemia may prove much more demanding in patients with specific pathologic conditions such as multiple insulinomas in the setting of multiple endocrine neoplasia type I (MEN-1), metastatic insulin-secreting carcinoma, or nesidioblastosis. The disease is then often multifocal or diffuse, and preoperative localization is rarely exhaustive. For these patients, a fine balance must be struck between removing total gross tumor and limiting prophylactic resection to prevent endocrine and exocrine pancreatic insufficiency.

For the last two decades, various insulin measurement techniques have been used for the management of insulinomas. Functional localization of insulinomas by measurement of insulin gradients after percutaneous venous sampling of the portal venous system was first described in 1975 by Ingemansson *et al.* in Surg. Gynecol. Obstet. 141 (1975), 705. Doppman *et al.* (Radiology 178, (1991), 237) further improved the potential of the functional localization of insulinomas by coupling intra-arterial calcium injection and insulin measurement in the suprahepatic veins (selective arterial calcium injection test).

Like most other endocrine peptides, insulin has a short half-life in the systemic circulation. Serum insulin levels decrease rapidly after resection of the oversecreting tissue. With the development of techniques for extemporaneous hormonal assay, intraoperative hormone measurements have been proposed to confirm the completeness of the surgical resection in various

endocrine diseases. Rapid radioimmunologic insulin assays have been described for almost two decades; however, practice has not led to incorporation of this measurement during surgical removal (intraoperatively) of insulinomas. In fact, in the medical practice insulin measurements are only retrospectively used.

In this light, it is noted that known insulin immunoassays, which are used in practice, consist of radioimmunoassays using polyclonal antisera which cross-react with proinsulins, and two-site assays using monoclonal antibodies. These immunometric assays have led to improvements in specificity and sensitivity as compared to radioimmunoassays. However, these known and practically used assays require specific laboratories, where the tests are carried out, and which, hence, require transportation of the samples to be tested and available measuring time and planning before test data are generated. The involvement of such specific clinical or chemical laboratories lead to the situation that the time between sampling and availability of the results in practice is more than 1 day.

The main aim of the present invention is, however, the provision of an insulin or C-peptide measurement that can be used prospectively, and hence intraoperatively.

In the World Journal of Surgery, 22 (1998) 1218-1224, Proye *et al.* describe that they have measured insulin intraoperatively during surgical management of insulinomas. Particularly, they describe that they have used intraoperative insulin measurements to guide the surgical treatment of various diseases, such as sporadic insulinomas, multiple endocrine neoplasia, insulin secreting carcinoma and pancreatic nesidioblastosis, diseases for which also the subject of the present invention is useful. More in detail, insulin was measured with a radioimmunologic assay in blood samples simultaneously drawn from a peripheral vein and the portal vein at the beginning of the operation and 20 minutes after the tumor removal. At the beginning of the procedure, after exposure of the portal vein and before any tissue was resected,

blood samples were drawn simultaneously from the portal vein and a peripheral vein. Then, standardized surgical explorations were performed, including exhaustive visual inspection and palpation from the inframesocolic area to the supramesocolic area. A Kocher's maneuver was performed and the posterior aspect of the pancreas tail was dissected. The whole pancreatic gland was subsequently manually palpated and further examined with intraoperative ultrasonography. Any detectable pancreatic tumor was then resected by enucleation or formal pancreatic resection. Twenty minutes after resection, simultaneous systemic and portal blood samples were drawn again.

The blood samples were tested on the serum levels of insulin using a radioimmunologic assay using polyclonal anti-insulin or anti-C peptide serum (Riagnost Insulin *ex* Hoechst-Behring) or a more specific immunoradiometric assay using monoclonal antibodies for insulin (Bi-Insuline IRMA *ex* Diagnostics Pasteur). The results of the tests were available within about 60 minutes.

By the nature of the test, this method requires that during the operation radiometric apparatuses outside the operating room need to be stand-by. Further, for an intraoperative assay 60 minutes is quite a long time, giving infection risks and so on. The present invention aims to solve these two problems in that it aims to provide a method that can be carried out in the operating room and that does not take that long.

Moreover, it would be highly desirable to have a method that makes it possible to determine or locate any excess insulin and/or C-peptide producing parts of the pancreas in a quantitative way, since that makes it possible to provide an alternative for or additional method to locate for instance insulinomas.

The above-identified aims are reached and the sketched problems are solved by the present invention. The present invention provides for a system that can be carried out on the spot. In addition, it provides a test method using equipment that can be moved or transported to each suitable

room without difficulties. It relates to a test procedure, wherein test results become available within about 20 minutes, i.e. it is possible to have the test results available within 20 minutes after blood samping; the test is real-time, reliable and provides the possibility of quantitative assessment of insulin, which makes the test really suitable for intraoperative applications. For example, it becomes possible to prospectively determine in which part of the pancreas an insulinoma, irrespective of its size, is located, and to determine intraoperatively whether the insulinoma(s) are removed.

More in detail, the present invention relates to a real-time test system comprising at least one reservoir with anti-insulin or anti-C peptide capture binders solidified in said reservoir, which reservoir is capable to receive a sample; optionally a wash solution; labelled anti-insulin or anti-C peptide binders useful as a tracer, wherein the label allows detection; and at least one detector.

The anti-insulin or anti C-peptide capture binders are for example and preferably selected from the group consisting of monoclonal anti-insulin or anti-C peptide capture antibodies; polyclonal anti-insulin or anti-C peptide capture antibodies; recombinant anti-insulin or anti-C peptide capture antibodies and anti-insulin or anti-C peptide capture antibodies obtained by Phage display technologies. The best mode and, hence, preferred embodiment, makes use of said monoclonal antibodies.

In a preferred embodiment, in the test system according to the invention the labelled anti-insulin or anti-C peptide binder or antobidy is present in dried form in the said reservoir. This embodiment requires less, or at least easier, handling than a method, wherein the tracer antibody is added in liquid form, right before the test or assay is to be carried out. It is noted that also this embodiment is within the scope of the present invention. In the system of the invention the reservoir preferably is a microtiter well. Moreover, since generally also standards and controls are used, the system of the invention is in the form of a microtiter well-plate.

As indicated, the present invention makes use of labelled tracer. This label, suitably, allowes for a photometric detection using a photomultiplier detector. Other detection systems that give suitable results are obtained when using colorimetric, fluorescent, enzymatic or other conventional labels.

In a preferred embodiment, the said labelled anti-insulin or anti-C peptide binders which preferably are monoclonal antibodies, are labelled by a chemiluminescent label, e.g. an acridinium ester, or an alkaline phosphatase/1,2-dioxetane system.

Immunoassays with chemiluminescence detection techniques have been increasingly accepted as platforms for immunoassay automation. Compared to other signal detection technologies, lower detection limits, and, therefore, a higher assay sensitivity, reduced background effects, and a large potential dynamic range of measurement can be achieved with chemiluminescent detection techniques. Moreover, signals can be amplified many-fold if coupled with an enzyme label such as horseradish peroxidase (HRP) or alkaline phosphatase. The combination of chemiluminescent detection and enzyme amplification has provided a very sensitive assay.

In a further aspect, the invention relates to a method for determining insulin and/or C-peptide levels in a sample, comprising adding the sample to a reservoir with anti-insulin or anti-C peptide capture binders solidified in said reservoir, and labelled anti-insulin or anti-C peptide binders useful as a tracer, followed by incubation giving labelled insulin complexes; optionally washing; and detecting the labelled insulin complexes photometrically. The washing step can be omitted in case a homogeneous test is carried out.

The insulin or C-peptide assay or test system of the invention allows for a direct and fast quantitative determination of the C-peptide insulin hormone. This fast test is real-time; it allows to give results in less than 30 minutes, and preferably even in less than 12 minutes. The system only

contains elements that make it possible that the test is carried out at the site where the test results are needed. The test system is hence in principle easily transportable.

By way of example, this assay may utilize two antibodies against different epitopes on the insulin molecule or of the C-peptide. Such monoclonal antibodies are commercially available and/or can be prepared using standard techniques: e.g. the C- and N-parts of insulin are separately used to immunisate mice; after sacrificing the mice spleen cells are fused with non-producing myeloma cells, followed by conventional selection, re-cloning to clonal, purification and concentration steps. One type of monoclonal anti-insulin or anti-C peptide antibody is attached to a solid surface in the reservoir, e.g. coated onto the surface of the reservoir, which preferably is a microtiter well. The other monoclonal antibody directed against another part of the insulin molecule is labelled, e.g. with a label that allows photometrical detection. The immunogenic substance can however also be the entire insulin or C-peptide molecule.

Specific antibodies for evaluation of glycated insulin in plasma and biological tissues are described by McKillop et al. in. J Endocrinol 2000 Oct; 167(1):153-63. They note that previous studies have shown that glycation of insulin occurs in pancreatic beta-cells under conditions of hyperglycaemia and that the site of glycation is the N-terminal Phe(1) of the insulin B-chain. To enable evaluation of glycated insulin in diabetes, specific antibodies were raised in rabbits and guinea-pigs by using two synthetic peptides (A: Phe-Val-Asn-Gln-His-Leu-Cys-Tyr, and B: Phe-Val-Asn-Gln-His-Leu-Tyr-Lys) modified by N-terminal glycation and corresponding closely to the N-terminal sequence of the glycated human insulin B-chain. For immunization, the glycated peptides were conjugated either to keyhole limpet haemocyanin or ovalbumin using glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester or 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride. Antibody titration curves, obtained using I(125)-tyrosylated tracer prepared from

glycated peptide A, revealed high-titre antisera in five groups of animals immunized for 8-28 weeks. The highest titres were observed in rabbits and guinea-pigs immunized with peptide B coupled to ovalbumin using glutaraldehyde. These antisera exhibited effective dose (median) (ED(50)) values for glycated insulin of 0.3-15 ng/ml and 0.9-2.5 ng/ml respectively, with negligible cross-reactivity against insulin or other islet peptides. The degree of cross-reaction with glycated proinsulin was approximately 50%. Glycated insulin in plasma of control and hydrocortisone-treated diabetic rats measured using rabbit 3 antiserum (1:10 000 dilution; sensitivity <19 pg/ml) was 0. 08+/-0.01 and 1.5+/-0.6 ng/ml (P<0.01), corresponding to 4 and 16% of total circulating insulin concentration respectively. Immunocytochemistry studies of the pancreas of streptozotocin-treated diabetic rats using a 1:1000 dilution of guinea-pig 2 antiserum revealed clusters of fluorescent positively stained cells in islets. These polyclonal antisera specific for glycated insulin are useful in the test system of the present invention.

Kao et al. disclose in Ann Clin Lab Sci 1992 Sep-Oct; 22(5):307-16 polyclonal antisera useful in a C-peptide test system. Thereto, two goats were immunized with synthetic C-peptide. Their antisera were immunopurified separately on a C-peptide affinity column. The purified antibodies from one goat were labeled with acridinium ester; the other goat's antibodies were immobilized on plastic beads. Standards were synthetic C-peptide. The new immunochemiluminometric assay (ICMA) for C-peptide was compared with routine radioimmunoassay (RIA) by using Novo's 125I-labeled C-peptide and Cambridge Medical's antiserum. Good correlations were found between the new ICMA and the RIA (r = 0.951; ICMA = 1.07 RIA + 147; n = 112). The ICMA showed good recoveries (91 percent to 108 percent) of added C-peptide and parallelism of diluted specimens.

Moreover, the molecular cloning, expression and mutagenesis of an anti-insulin single chain Fv (scFv), is described by Lake et al. in Mol Immunol 1994 Aug; 31(11):845-56.

The immunoglobulin variable region genes of a murine anti-insulin IgG-producing hybridoma were rescued and cloned into a bacterial expression vector. The variable regions of the gamma heavy chain and the kappa light chain were expressed independently and together as a single chain antibody (scFv). The variable heavy chain alone demonstrated the ability to bind to insulin. The kappa light chain did not show any binding activity towards insulin. The scFv was constructed by PCR assembly using a (Gly4Ser)3 linker between the carboxyl end of the variable heavy chain and the amino terminus of the kappa light chain. The scFv bound insulin at an IC50 of 3.5 x 10(-8) M whereas the parent antibody bound insulin at 1.0 x 10(-8) M. Mutagenesis of the variable heavy chain complementarity determining regions (CDR) indicated that CDR1 and CDR3 were important for binding to insulin. Position 99 in CDR3 of the heavy chain was found to be a critical position for the ability of the scFv to bind to insulin.

The above techniques can give suitable binders for use in the test system of the present invention.

The insulin or C-peptide assay of the present invention preferably is a one step, two-site chemiluminescent immunometric assay. Suitable systems are based on the well-known accusphere technology of Organon Teknika. In a preferred embodiment the second antibody is labelled with isoluminol. The labelled second antibody, preferably is present in a dry form in the reservoir. More in particular, the test system of the present invention makes use of a microtiter strip plate that is ready to use.

In use, generally, first a standard curve is made. To check the validity of this standard curve controls are measured. When the controls are within the indicated area, the system is ready to receive and measure samples such as serum, EDTA plasma, perfusion liquid, whole blood or cell medium or culture supernatant.

A suitable alternative is a system using a master curve. The test manufacturer provides a master curve which by means of one or more

calibrators can be converted in a so-called working curve. The working curve allows reading of unknown samples.

In the standard curve technique, the insuline and/or C-peptide containing samples are introduced in the binder or antibody containing reservoir. Preferably, this is done by means of a multi-channel pipette. The sample, standards and controls are incubated for a short predescribed time, in the preferred embodiment 7 minutes at room temperature. The incubation is generally carried out while shaking the reservoirs, for instance on a STAT-Shake (*ex* Future Diagnostics B.V., Wijchen, The Netherlands). During this incubation step sandwich complexes are formed. Unbound-labelled antibody is removed by a wash step, for instance with a PBS wash buffer, while using a STAT-Wash (*ex* Future Diagnostics B.V., Wijchen, The Netherlands). The washing step is followed by the detection protocol. When the label is a chemiluminescent label, this generally encompasses the use of activator solutions. In a preferred embodiment, the label is luminol and the activation makes use of a sodium hydroxide solution (4%), and a peroxide solution (0.12%). For this activation step suitable use can be made of a STAT-Read (*ex* Future Diagnostics B.V., Wijchen, The Netherlands) which automatically injects two activator solutions, initiating the chemiluminescence reaction. The amount of label is detected using a photometrical method, generally involving a photomultiplier. The time between obtaining a blood sample and reading a result can be as low as about 10 minutes.

Suitable detectors are for instance:

- *the Beckman Coulter Access.*

It is an automated, continuous random access immunoassay system which employs a paramagnetic particle solid phase to separate free and bound analyte and an alkaline phosphatase-mediated chemiluminescent reaction for signal detection. Lumi-Phos 530, a dioxetane-based chemiluminescent substrate, is dephosphorylated upon the addition of alkaline phosphatase, resulting in release of light. The light emitted is measured by a luminometer.

- *The ACS:180 SE by Chiron Diagnostics.* It uses direct label chemiluminescence technology. The chemiluminescent agent, acridinium ester is coupled to antibodies or antigens. The release of light upon addition of hydrogen peroxide, nitric acid solution, and sodium hydroxide to bound components of the reaction is detected by a luminometer.

- *The ACS Centaur by Chiron Diagnostics.*

- *The DPC Immulite and Immulite 2000.* Immulite immunoassays employ enzyme-amplified chemiluminescence labeling. Alkaline phosphatase is conjugated to analyte specific antibodies for antibody excess immunometric assays or to analytes or biotinylated analytes for antibody limited competitive assays. The chemiluminescent substrate for alkaline phosphatase is 1,2-dioxetane, which is destablized by alkaline phosphatase leading to an excited anion intermediate. The unstable dioxetane intermediate will emit light upon decay back to the ground state. The Immulite assay tubes contain one polystyrene bead providing a solid phase for analyte-specific antibody, and serves as the reaction vessel for incubations, washes, and signal development. Also Immulite 2000 uses antibody or antigen coated polystyrene beads as a solid phase. It uses alkaline phosphatase or acridinium ester labels.

- *The Vitros ECI by Johnson & Johnson Clinical Diagnostics.* The Vitros ECi utilizes streptavidin-coated wells for heterogenous immunoassay reactions. The Vitros ECi employs enhanced chemiluminescent detection technology in which HRP is conjugated to the antibody. An enhancer is oxidized by the HRP to an unstable radical intermediate that, in turn, oxidizes the reaction substrate luminol to an electronically excited state aminophthalate product that emits sustained light when returning to the ground state.

- *The Elecsys 1010 and 2010 by Roche Diagnostic Systems.* In electrochemiluminescence, reactive species are generated from stable precursors, such as compounds of ruthenium, at the surface of an electrode. Emission of light from the ruthenium is initiated electrochemically via reaction of the ruthenium species with tripropylamine. In the instrument's

measuring cells, magnetic particles with bound label are deposited on the electrode using a magnet followed by removal of excessive sample. A voltage is applied to initiate the electrochemil-uminescent reaction and light emission measured with a photomultiplier tube. Following completion of the reaction, the magnetic beads are released and the well washed. The electrochemiluminescent technique eliminates problems associated with reagent addition, and the fluid phase reaction with no mixing allows for shorter incubation times. Use of the low molecular mass ruthenium marker that allows signal amplification significantly enhances sensitivity and increases linear range.

- *The Architect i2000 by Abbot.* The Abbott Architect i2000 is a new generation, fully automated, chemiluminescent immunoassay analyzer with a modular design for system flexibility. The system performs solid phase chemiluminescent immunoassays. An acridinium sulfonamide conjugate is used for signal detection.

- *The Advantage by Nichols Institute Diagnostics.* Steptavidin-coated magnetic particles and biotinylated antibodies are employed in the assay system. Acridinium ester is the chemiluminescence label for signal detection.

This fast, preferably one step insulin assay offers a quick, reliable, and quantitative result of insulin levels and may be used as an intra-operative tool in the localization of, for instance, insulinoma tumors in the pancreas and monitoring the effectiveness of surgical treatment of insulinomas.

By way of example, the present invention is now described in detail for the localization and removal of insulomas. In this light, it is noted that the pancreas body and tail have a limited number of connections to the *Vena splenica*, whereas the neck and head of the pancreas additionally also have a limited number of connections to the *Vena porta*. An insulinoma is a tumor that hypersecretes insulin sometimes up to 10,000 times the normal production. By determining the insulin content of blood samples taken at different spots in the *Vena splenica* and *Vena porta*, the medical professional

can within 20-30 minutes dependent on the influx of insulin in the *Vena splenica* and *Vena porta* easily and accurately determine where an insulin secreting tumor is situated in the pancreas. Moreover, the invention makes it possible that after the enucleation or resection, the surgeon can on the basis of an additional sampling in one of or both the *Vena splenica* and *Vena porta* within 20-30 minutes determine whether the operation can be completed or that there are still hyperexcreting tumor cells left.

The samples can, e.g. and by preference, be taken while using a probe that can be brought in the *Vena splenica* and *Vena porta* for instance from the groin, and that is arranged in such a way that blood samples can be collected.

In a particular aspect, the present invention, hence, also relates to a method for determining insulin levels, comprising sampling blood in the *Vena splenica* and/or *Vena porta*, comprising the steps of introducing a probe in one of said veins, sampling at one or more spots in the said vein(s), and analysing the samples using the above-described general method of the invention.

In a preferred embodiment, the present invention relates to a system comprising a probe arranged to take samples from veins in the body; and the test system of the invention. In particular, this system comprises a probe arranged to be introduced in the *Vena splenica* and/or *Vena porta*.

The subject of the present invention can also be used as a quick, real-time insulin test when a decision is to be made on whether a particular pancreas can be used for transplantation.

At present, in case of a transplantation a pancreas available for transplantation must be brought in a patient's body within 6 hours after removal from the donor. In these 6 hours, the pancreas must often be transported, and subjected to a number of tests. One of the tests encompasses perfusion of the pancreas, followed by measurement of the insulin production in the perfusion liquid. The test is carried out in specialized laboratories, and on average the test results are available within about 24 hours. At that time,

the pancreas is already placed in the patient's body, which makes that if the test gives negative or unfavourable results in a worst case scenario a second operation may be needed to remove the transplanted pancreas.

The assay and techniques of the present invention make it possible to have the test data available on the spot and within less than 20 minutes, preferably in about 10 minutes. The test data can hence be used to take the decision whether or not the transplantation should be carried out.

Until the moment of the present invention, wide-scale use of insulin assays was a subject of research rather than a diagnostic application. Spontaneous hypoglycaemia, a disorder which can be caused by hyperinsulinism, insulinoma, insulin autoimmune syndrome and non-insulin-mediated factors, was almost the only clinical indication for the measurement of plasma insulin. Diabetes mellitis is diagnosed solely on the basis of chronic hyperglycaemia. Thus, measurement of plasma insulin has no clininal value in the diagnosis or management of diabetic patients, with the exception of rare cases, including the syndrome of severe insulin resistance and abnormalities in beta-cell secretory products. Otherwise, insulin measurement is used in experimental investigations to study the pathophysiology of various disorders, especially diabetes.

However, with the provision of the present invention such insulin tests become practically usable in diagnosis. For instance, the assay and method of the invention is of practical use is in the provision of individual protocols for patients suffering from diabetes mellitis. More in particular, the quick test of the invention makes it possible that for patients for which diabetes mellitis was diagnosed, the need of insulin can be determined on the basis of the reaction of the body to the intake of carbohydrate and in particular sugar containing food. This reaction of the body, of course, is patient dependent. To determine reference values and limits, insulinaemia must be measured in normoglycaemic subjects with a normal body weight. Moreover, as insulinaemia is most often measured during stimulation tests, reference

values must also be determined for the most common tests such as the oral glucose tolerance test or the intravenous glucose tolerance test.

The present invention makes it possible to have for each individual patient a very specific protocol for stimulation tests can be carried out within 1 or 2 days to determine the optimal insulin requirements. Thereto, blood samples taken before and after the uptake of a particular food are analysed for insulin using the assay of the invention. Moreover, the present invention allows that a medical professional is no longer required. The assays can be carried out by an assistant.

In a further aspect, the present invention is practical in use in a method wherein beta cells, generally of human or porcine origin, are cultured *in vitro* or *ex vivo*. The beta cells are monocellular and should be well-producing in respect of insulin. The aim of these cultured beta cells is to be implanted in the pancreas of a patient suffering from hypoinsulaemia. The present invention allows to check right before the implantation whether the cultured beta cells after transportation to the medical professional who carries out the implantation still have the required insulin producing activity. The medical professional can determine at the spot whether suitable beta cells are obtained. Moreover, the activity of the beta cells after implantation can be checked by the present invention as well.

More in particular, it is noted that regulatory challenges for manufacturing islets for transplant application include the definition of prospective product release criteria, which would allow appropriate pre-transplant islets potency assessment. Conventional kits for measurement of insulin levels folling *in vitro* assays of glucose-stimulated insulin release require between 3 and 24 hours. The immunoassay of the invention, and preferably the chemiluminescent immunoassay, provides results within 10 minutes. This minimization of assay time is highly desirable to reduce the *in vitro* culture period before transplantation while identifying poorly functioning islet preparations in a timely fashion. The islets to be transplanted may, if

needed, be used together with suitable compounds or programs to avoid rejection. After transplantation of the beta-cells the measurement of C-peptide can be done, to check the effectiveness of the transplantation. Insulin is not suitable to be measured at this stage since the patients will still need to admit themselves with exogenous insulin.

The present invention will now be further illustrated while referring to the following, non-limiting examples.

Example 1 (Best Mode)

Components in the preferred insulin C-peptide assay kit of the invention:

1. Ready To Use Strip Plate (P1):

A microtiter strip plate (32 wells) is coated with monoclonal anti-insulin or anti-C peptide as capture antibody. An isoluminol labelled monoclonal anti-insulin or anti-C peptide antibody is present in the well as a tracer antibody in dried form.

2. Insulin C-peptide Zero Standard (SO):

1 vial labelled 'SO' contains lyophilized insulin-free human serum albumin.

3. Insulin C-peptide Standards (S1, S2, S3, S4, S5):

Vials labeled 'S1" through 'S5' contain a standard amount insulin of C-peptide in a C-peptide- or insulin-free human serum albumin in lyophilized form. The standards can, for instance calibrated against the 1st WHO International Reference Preparation (IRP) of Insulin (code 66/304).

4. Insulin C-peptide Controls (C1 and C2)

Vials labeled 'C1' (Control 1) and 'C2" (Control 2) contain a controlled amount of insulin or C-peptide in an insulin-free human serum albumin in lyophilized form.

5. PBS Wash buffer (10 x concentrated) (W 1)

One vial labeled 'W1' contains 30 mL of 10 times concentrated phosphate buffered saline with 0.09% sodium azide as a preservative.

6. Non-coated Strip Plate (P2):

A microtiter strip plate (32 wells), with natural colored non-coated wells for the purpose of collecting and aliquoting of standards, controls and patient plasma samples.

7. Activators (A1 and A2):

Two bottles labeled 'A1' (Activator 1) and 'A2' (Activator 2) each contain 20 mL Activator Solutions to generate the chemiluminescence light signal. Activator 1 (A1) contains 4% sodium hydroxide (NaOH). Activator 2 (A2) contains 0.12% peroxide.

Additional materials and instruments required

1. Precision pipettes: 150 µl and 1000 µl
2. Distilled or deionized water
3. Timer
4. EDTA collection tube
5. Serum collection tube
6. Future Diagnostics STAT-Centrifuge; STAT-Shake; STAT-Wash; and STAT-Read
7. Measuring glass of 300 ml

Reagent preparation and storage

1. The Ready to Use Strip Plate (P1) should be at room temperature;
2. Insulin Zero Standard (SO) is reconstituted in the vial labelled 'SO' with 2.0 ml of distilled or deionized water. The vial is allowed to stand 15 minutes

at room temperature, then mixed by gentle shaking to ensure complete reconstitution;

3. Insulin C-peptide Standards (S1 - S5) are reconstituted in the vials labelled 'S1' through 'S5' with 1.0 ml of distilled or deionized water. The vials are allowed to stand 15 minutes at room temperature, then mixed by gentle shaking to ensure complete reconstitution;

4. Insulin C-peptide Controls (C1 and C2) are reconstituted in the vials labelled 'C1' and 'C2' with 1.0 ml of distilled or deionized water. The vials are allowed to stand for 15 minutes at room temperature, then mixed thoroughly by gentle shaking to ensure complete reconstitution;

5. Wash Solution (W1) is diluted with 270 ml of distilled or deionized water and mixed;

6. Non-coated Strip Plate (P2) is sampled;

7. Activator Solutions (A1 and A2) should be stored at 15-30°C until expiration date on vial;

The determination of Insulin or C-peptide should be performed in serum, EDTA plasma, in perfusion solution or in cell-culture supernatent. It is recommended to perform the assay in duplicate, for this purpose at least 500 µl of patient sample perfusion solution or cell-culture supernatant is needed.

1. Proper sample collection from the patient requires one, non-diluted blood sample to be drawn in glass tubes with EDTA as anticoagulant (i.e. Lavender top Vacutainer, Becton Dickenson or equivalent) and is shaken head over tail for 5 times to ensure proper anticoagulant mixing. Alternative for serum the blood should be collected in a Vacutainer (no additives). Separate either plasma or serum from cells as soon as possible, by means of centrifugation (10 min. at 2000 g-force).
2. If the plasma or serum is analyzed at a later date the plasma or serum sample should be frozen immediately.

3. Just prior to setting up the insulin assay the frozen patient samples must be thawed.

The insulin C-peptide test procedure

Procedure

- Label the micro titer strips, 1 to 4, of the non-coated strip plate 'P2' and pipette in duplicate in vertical sequence of the wells (A1-H1)(A2-H2) etc., 150 µl of: Zero Standard (SO), Standards (S1-S5), Controls 1 and 2 (C1, C2), and patient plasma sample.

- Label the microtiter strips, 1 to 4, of the ready to use strip plate 'P1", identical to the labelling of the non-coated strip plate 'P2'.

- Transfer with the a 100 µl pipet the appropriate Standards, Controls and patient samples from the non-coated strip plate 'P2', directly into the bottom of the wells of the ready to use strip plate 'P1'.

- Place the ready to use strip plate, 'P1' on the STAT-shake immediately and incubate for exactly 7 minutes at room temperature.

- Wash the strips in the STAT-Wash using 300 µl of diluted wash solution. Repeat 2 times.

- The washed strips are measured in the STAT-Read within 15 minutes after washing of the strips.

- Count each micro titer strip for 3 seconds in the STAT-Read using Activator A1 and Activator A2 respectively, 100 µl each. The entire

chemiluminescence reaction is complete in 3 seconds. This reaction is irreversible, so each well can only be read once.

- Patient samples with values greater than the highest standard (S5) may be diluted with Zero Standard (SO) and re-assayed. Result values must be multiplied by the dilution factor.

The insulin assay of the invention allows for an analytical sensitivity of 4 pmol/L and a dynamic range up to 2000 pmol/L. The intra-assay variation at 65, 359 and 1029 pmol/L are 5.2%, 8.2% and 6.7% respectively. Inter-assay variation at 37, 212 and 632 pmol/L are 9.1%, 9.3% and 10.7% respectively. The mean per sample of high-low recovery and parallelism are within 83-103%. The cross reactivity with Glucagon, C-peptide and Pro-Insulin was < 0.5% and no high dose hook was observed at concentrations > 200,000 pmol/L.

The Insulin assay was compared to a commercially available Insulin Enzyme Immuno Assay (EIA), on a population of 41 samples. The range of values obtained using the commercially available insulin EIA (A) ranged from 11.2 to 751 pmol/L. Values obtained with the Insulin Assay (B) ranges from 16.5 to 1232 pmol/L. A correlation coefficient of (r) = 0.99 with a regression formula Y = 1.72X - 4.2 was obtained from linear regression analysis of the data.

The C-peptide assay of the invention allows for an analytical sensitivity of <5 pmol/L and a dynamic range up to 7000 pmol/L. The inter-assay variation at 570, 1900 and 3700 pmol/L are 11.9%, 7.9% and 10.6%, respectively. The mean per sample of spike recovery and parallelism are within 83-123%.

The STAT-C-peptide assay was compared to a commercially available C-peptide. Enzyme Immuno Assay (EIA), on a population of 36

samples. The range of values obtained using the commercially available C-peptide EIA (A) ranged from <15 to 1469 pmol/L. Values obtained with that STAT-C-peptide assay (B) ranged from <5 to 2886 pmol/L. A correlation coefficient of (r) = 0.93 with a regression formula Y = 1.89X - 27 was obtained from linear regression analysis of the data.

Example 2

In 1994, a patient suffered from collapses appearing temporarily during the day and losses of consciousness that manifested as a loss of sight and speaking ability lasting for seconds. He underwent a blind insulinoma resection.

In order to find the tumor which caused the hypersecretion of insulin several ultrasonographs (USG) and CT scans have been performed. No focal changes have been found. The decision to perform the operation without localization was made.

No focal changes were found during the operation. The decision to perform "blind" resection of the body and tail of the pancreas was made. Although the symptoms were less severe, the patient was not cured. Five months after the operation the patient started to take diazoxyde at a dose of 2 x 100 mg. This dose went up to 600 mg per day.

In 2000, that patient suffered from general malaise appearing temporarily and losses of consciousness lasting for seconds and appearing 2-3 times a month. Upon testing, he showed elevated insulin levels.

To locate the tumor, USG, CT scan and ASVS (Arterial Stimulating and Venous Samping) was performed. The results of all these examinations were negative. The decision of re-operation was made.

At the beginning of the operation, a blood sample was taken from a peripheral vein and the insulin level was measured with the insulin assay of of example 1. The result was 84 pmol/l. Then, the pancreatic gland was examined very carefully after having done the Kocher's maneuver. Neither visible nor

palpable focal changes were found. More insulin levels were measured intraoperatively using the assay of the invention. Blood samples were taken from the mesenteric superior vein at the lower edge of the pancreas, the mesenteric superior vein in the middle of the gland, the portal vein and splenic vein. The results were: 832 pmol/l, 463 pmol/l, 302 pmol/l and 106 pmol/l insulin, respectively. Intraoperative ultrasound examination confirmed the presence of occult tumor located in the middle part of the head of pancreas. The tumor was removed, then the insulin levels from the places mentioned above were measured again - to confirm that the removed tissue was responsible for the hypersecretion of insulin. The results were 68 pmol/l, 70 pmol/l, 73 pmol/l and 103 pmol/l, respectively. Intraoperative pathological examination revealed that the tumor was an *insulinoma*. Considering the fact that the post-excision insulin levels were much lower than the pre-excision insulin levels, the presence of another tumor was rather unlikely. The decision to end the operation was made.

After the procedure, temporary hyperglycemia occurred, but the infusions of insulin were not necessary. Normoglycemia was achieved within 3 days. In the postoperative course, pancreatic fistula and then subphrenical abscess appeared. Both these complications were treated operatively. Glucose levels were within normal limits and no symptoms of hypoglycemia were observed.

Example 3

As indicated in the general description (*vide supra*), now a surgeon's judgement of a successful pancreas transplantation is the return of a typical pink color of the transplanted pancreas and the ability of the transplanted pancreas to lower the glucose level. While using the assay of the present invention, prior to the transplantation, the well-functioning of the pancreas can be tested by measuring the insulin-level in the perfusion solution upon stimulating the pancreas with increasing concentrations of glucose. This is hard to quantify because each pancreas would react differently to the added

dose of glucose. However, as a response to the stepwise addition of glucose the well-functioning pancreas should show a comparable stepwise increase in insulin secretion into the perfusion solution.

After the successful transplantation the surgeon can measure peripherally the concentration of insulin (or C-peptide for that matter), which should preferably be within normal range (8-20 mIU/liter) dependent on the level of glucose in the patients blood system.

Example 4

With the assay of the invention, prior to implantation of cultured beta cells, the supernatant of the culture can be measured at the spot for insulin levels (or C-peptide levels) upon treating the cell culture with glucose. The level of secreted insulin depends on the concentration of beta cells in the culture. Properly producing beta cells should show a dose-dependent secretion.

Human islets were isolated and purified on Eurocollins-Ficoll gradients. Aliquots of fifty islets (n=4, n=6, n=3) obtained from three consecutive human donor pancreate were subjected to low (40 mg/dl), high (400 mg/dl), and low glucose buffer for a period of one hour. One milliliter of supernatant was collected from each aliquot at the end of the hour incubation period. Insulin levels were simultaneously measured by standard ELISA and by the Immunoassay described in Example 1. Raw insulin values and stimulatin index from each aliquot were compared.

Comparing the results of these assays, the correlation for the raw insulin data was 0.99 and for the stimulation index was 0.96.

The Immunoassay of the present invention is a reliable method for rapid assessment of insulin production from isolated human islets. This assay is, hence, of assistance for the development of potency product release criteria allowing minimization of the time-gap between isolation and transplantation.

After the successful implantation the surgeon can measure peripherally the concentration of insulin (C-peptide), which should be

preferably within normal range (8-20 mIU/liter), dependent on the level of glucose in the patients blood system.

Example 5

With diabetes patients the glucose tolerance can be tested by measuring the immediate response (and in time) of the insulin levels, while using the assay of the invention.

Example 6

Also real-time measurements upon the intake of oral beta-cell stimulating drugs can be carried out to fine-tune the concentration of the drugs, at the spot while using the assay of the invention.

Claims

1. Real-time test system comprising at least one reservoir with monoclonal anti-insulin or anti-C peptide capture binders solidified in said reservoir, which reservoir can receive a sample; optionally a wash solution; labelled anti-insulin or anti-C peptide binders useful as a tracer, wherein the label allows detection; and at least one detector.
2. The test system according to claim 1, wherein the anti-insulin or anti C-peptide capture binders are selected from the group consisting of monoclonal anti-insulin or anti-C peptide capture antibodies; polyclonal anti-insulin or anti-C peptide capture antibodies; recombinant anti-insulin or anti-C peptide capture antibodies obtained by Phage display technology.
3. The test system of claim 1 or claim 2, wherein said anti-insulin or anti-C peptide capture binders are monoclonal anti-insulin or anti-C peptide capture antobides.
4. The test system of any of the preceding claims, wherein the labelled monoclonal anti-insulin or anti-C peptide is present in dried form in the said reservoir.
5. Test system according to any of the preceding claims, wherein the said labelled anti-insulin or anti-C peptide capture binders are labelled by a label selected from the group consisting of chemiluminescent labels, colorimetric labels, fluorescent labels and enzymatic labels.
6. The system of claim 1, wherein the reservoir is a microtiter well.
7. A method for determining insulin or C-peptide levels in a sample, comprising adding the sample to a reservoir with monoclonal anti-insulin or anti-C peptide capture binders solidified in said reservoir, and labelled anti-insulin or anti-C peptide binders useful as a tracer, followed by incubation giving labelled insulin or C-peptide complexes;optionally washing; and detecting the labelled insulin or C-peptide complexes.

8. The method of claim 7, wherein the detection is a photometrical detection.
9. The method of claim 7, wherein the sample is perfusion solution obtained from a pancreas removed from the body after stimulating said pancreas with an insulin-production influencing compound, preferably glucose.
10. The method of claim 7, wherein the sample is supernatant of *in vitro* cultured beta cells.
11. The method of claim 7, wherein the sample is a blood sample.
12. A method for determining insulin and/or C-peptide levels, comprising sampling blood in the *Vena splenica* and/or *Vena porta*, comprising the steps of introducing a probe in one of said veins, sampling at one or more spots in the said vein, and analysing the samples using the method of claim 7.
13. System for carrying out the method of claim 12, comprising a probe arranged to be introduced in the *Vena splenica* and/or *Vena porta* and the test system of any one of claims 1-6.

INTERNATIONAL SEARCH REPORT

PCT/NL 02/00183

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/74 G01N33/574 G01N33/533

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBASE, MEDLINE, CHEM ABS Data, BIOSIS, EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 104 029 A (MAIER JR CHARLES L) 1 August 1978 (1978-08-01) cited in the application abstract claims 1,2	1-13
X	EP 0 484 961 A (TOSOH CORP) 13 May 1992 (1992-05-13) cited in the application abstract	1-13
X	WO 98 59246 A (PAU BERNARD CHRISTIAN ;KAMAL NADIA (FR); BOUANANI MAJIDA (FR); LAR) 30 December 1998 (1998-12-30) cited in the application abstract claims 1-14	1-13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2002	23/07/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PROYE, C. ET AL.: "Intraoperative Insulin Measurement during Surgical Management of Insulinomas" WORLD JOURNAL OF SURGERY, vol. 22, no. 12, 1998, pages 1218-1224, XP001058267 cited in the application page 1219, left-hand column, paragraph 3 -right-hand column, paragraph 2 ---	1-13
A	SCHULZ B ET AL: "INTERRELATIONSHIPS BETWEEN PORTAL AND PERIPHERAL VENOUS INSULIN PRO INSULIN AND GLUCAGON CONCENTRATIONS AFTER ORAL GLUCOSE ADMINISTRATION IN MAN" ENDOKRINOLOGIE, vol. 68, no. 3, 1976, pages 309-318, XP001051058 ISSN: 0013-7251 the whole document ---	1,13
A	AGARWAL A ET AL: "Intraoperative ultrasonography for insulinoma: a preliminary experience." INDIAN JOURNAL OF GASTROENTEROLOGY, (1997 APR) 16 (2) 58-9., XP001058463 the whole document ---	1,13
A	WANG J ET AL: "NEEDLE-TYPE DUAL MICROSENSOR FOR THE SIMULTANEOUS MONITORING OF GLUCOSE AND INSULIN" ANALYTICAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. COLUMBUS, US, vol. 73, no. 4, 15 February 2001 (2001-02-15), pages 844-847, XP001060093 ISSN: 0003-2700 page 844, right-hand column page 845, left-hand column, paragraph 3 -right-hand column, paragraph 2 -----	1-13

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 12 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

INTERNATIONAL SEARCH REPORT

PCT/NL 02/00183

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: -
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/NL 02/00183

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4104029 A	01-08-1978	US 4181650 A	01-01-1980
EP 0484961 A	13-05-1992	JP 3044569 B2	22-05-2000
		JP 4177166 A	24-06-1992
		DE 69127255 D1	18-09-1997
		DE 69127255 T2	04-12-1997
		EP 0484961 A1	13-05-1992
WO 9859246 A	30-12-1998	FR 2764989 A1	24-12-1998
		AU 8219198 A	04-01-1999
		EP 0995121 A1	26-04-2000
		WO 9859246 A1	30-12-1998
		JP 2002511143 T	09-04-2002
		ZA 9805374 A	20-12-1999

专利名称(译)	缩短周转时间胰岛素或c肽测定		
公开(公告)号	EP1370873A1	公开(公告)日	2003-12-17
申请号	EP2002707334	申请日	2002-03-21
[标]申请(专利权)人(译)	FUTURE诊断		
申请(专利权)人(译)	FUTURE诊断B.V.		
当前申请(专利权)人(译)	FUTURE诊断B.V.		
[标]发明人	MARTENS MICHAEL FRANCISCUS WILHELMUS CORNELIS ROSMALEN FRANCUSCUS MARIA ANNA		
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优先权	2001201107 2001-03-23 EP 09/850363 2001-05-07 US		
外部链接	Espacenet		

摘要(译)

本发明涉及用于分析胰岛素或C-肽样品的方法的实时测试系统。特别地，本发明使得可以在需要测试结果的位置附近进行分析。本发明特别适用于术中和糖尿病相关（即β细胞移植）应用。本发明可用于$ i（in）和$ i（离体）应用。