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(54) **IMPROVED IMMUNODIAGNOSTIC ASSAYS USING REDUCING AGENTS**

VERBESSERTES IMMUNDIAGNOSTISCHE TESTS DURCH VERWENDUNG VON REDUZIERENDE AGENZIEN

AMELIORATION DE DOSAGES D'IMMUNODIAGNOSTIC PAR UTILISATION D'AGENTS REDUCTEURS

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## Description

### FIELD OF THE INVENTION

**[0001]** The present invention relates to the field of diagnosis and treatment of HCV infection. More particularly, the present invention relates to HCV NS3 helicase and its uses. Also the present invention relates to improved immunodiagnostic assays.

### BACKGROUND OF THE INVENTION

**[0002]** Hepatitis C Viruses (HCV) constitute a genus within the Flaviviridae, with closest homology to the hepatitis G and GB viruses, and Pestiviruses. The positive-stranded RNA genome encodes at least 9 proteins. Core, E1, and E2 constitute the structural proteins. NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural (NS) proteins. HCV isolates display high levels of sequence heterogeneity allowing classification into at least 11 types and 90 subtypes (Maertens and Stuyver, 1997). HCV infection of the human liver is often clinically benign, with mild icterus in the acute phase. The disease may even go unnoticed in some cases of acute resolving hepatitis C. In the majority (>70%) of cases, however, HCV infection leads to chronic persistent or active infection, often with complications of liver cirrhosis and auto-immune disorders. Hepatocellular carcinoma may occur after about 20 to 35 years (Saito et al., 1990), sometimes even without the intermediate phase of cirrhosis. No prophylaxis is available today and treatment with interferon-alpha (IFN-α) only leads to long-term resolution in about 4 to 36% of treated cases, depending on the HCV genotype (Maertens and Stuyver, 1997).

**[0003]** Since productive culture methods for HCV are currently not available, and since only minute amounts of HCV antigens circulate in the infected patient, direct detection of HCV particles cannot be performed routinely, and indirect diagnosis is only possible using cumbersome amplification techniques for HCV RNA detection. Unlike with many other viral infections, HCV particles generally persist in the blood, liver, and lymphocytes despite the presence of cellular and humoral immune response to most of the HCV proteins HCV antibodies can be conveniently detected by Elisa techniques which allow high throughput screening in blood banks and clinical. laboratories. Supplementary antibody testing is required and is now mandatory in most countries. True HCV reactivity is thus discriminated from false reactivity, which may be caused by non-specific binding of serum or plasma itnmunoglobulines or anti-idiotypic components to the coating or blocking reagents, or to contaminants present in HCV antigen preparations, or even to fusion parts or non-specific regions of the recombinant antigens themselves (McFarlane et al., 1990). HCV RNA detection by PCR or branched DNA (bDNA) techniques have recently been introduced to monitor chronic HCV disease, especially during therapy. Surprisingly, HCV RNA detection is sometimes employed to confirm HCV Ab screening tests, despite the fact that only ~70-94% of repeatedly HCV Ab positive patient samples are positive by nested PCR (Marin et al., 1994). Of HCV Ab positive blood donors, who usually present with milder forms of the disease and low HCV RNA levels, confirmation by nested PCR is usually in the order of ~40% (Waumans et al., 1993; Stuyver et al., 1996). Strip-based assays therefore provide the only reliable alternative for HCV Ab confirmation. Even in the case of an indeterminate result in the confirmatory assay, serological follow up of the patient rather than HCV RNA detection is advisable (Di Bisceglie et al., 1998). Since native HCV antigens are not available in sufficient quantities, such confirmatory assays incorporate synthetic peptides and/or recombinant fragments of HCV proteins. One of the most critical issues in the confirmation of antibodies constitutes the reactivity of the NS3 protein (Zaaijer et al., 1994). NS3 antibodies often appear first in seroconversion series and the reactivity of the NS3 protein seems to be different in the different commercial assays available today . EP 0 139 526 describes an HCV NS3 protein reacted with an aldehyde and incubated in the presence of a reducing agent and a method for preparing a solid phase immunoassay using said HCV NS3 protein.

**[0004]** Innogenetics introduced the concept of strip technology in which usually a combination of synthetic peptides and recombinant proteins are applied as discrete lines in an ordered and easily readable fashion. The INNO-LIA HIV Ab tests have proven to be superior to routinely used western blots (Pollet et al., 1990). The Line Immuno Assay allows multiparameter testing and thus enables incorporation of cutoff and other rating systems, sample addition control, as well as testing for false reactivity to non-HCV proteins used as carrier or fusion partner required for some antigens in the Elisa test. In principle, the test format allows to combine antigens of different aetiological agents or phenotypically linked conditions into a single test.

**[0005]** The INNO-LIA HCV Ab III is a 3rd generation Line Immune Assay which incorporates HCV antigens derived from the Core region, the E2 hypervariable region (HVR), the NS3 helicase region, and the NS4A, NS4B, and NSSA regions. In the third generation assay, highly. purified recombinant subtype 1b NS3 protein and E2 peptides enabled superior sensitivity while safeguarding the reliable specificity which is characteristic of peptide-based tests (Peeters et al., 1993). Perhaps one of the most important features of this assay is its unprecedented correlation with HCV RNA positivity (Claeys et al., 1992; De Beenhouwer et al., 1992).

**[0006]** The antigens are coated as 6 discrete lines on a nylon strip with plastic backing. In addition, four control lines

are coated on each strip: anti-streptavidin, 3+ positive control (anti-human Ig), 1+ positive control (human IgG), and the ± cutoff line (human IgG). A diluted test sample is incubated in a trough together with the LIA III strip. If present in the sample, HCV antibodies will bind to the HCV antigen lines on the strip. Subsequently, an affinity-purified alkaline phosphatase labelled goat anti-human IgG (H+L) conjugate is added and reacts with specific HCV antigen/antibody complexes if previously formed. Incubation with enzyme substrate produces a chestnut-like color, the intensity of which is proportionate to the amount of HCV-specific antibody captured from the sample on any given line. Color development is stopped with sulphuric acid. If no HCV-specific antibodies are present, the conjugate only binds to the ±, 1+, and 3+ control lines. If the addition of sample is omitted, only the ± and 1+ control lines will be stained.

## DEFINITIONS

**[0007]** The following definitions serve to illustrate the different terms and expressions used in the present invention.

**[0008]** The term 'HCV NS3' protein' refers to a polypeptide or an analogue thereof (e.g. mimotopes) comprising an ammo acid sequence (and/or amino acid analogues) defining at least one HCV epitope of either HCV NS3 protease or helicase.

**[0009]** The term 'hepatitis C virus envelope protein' refers to a polypeptide or an analogue thereof (e.g. mimotopes) comprising an amino acid sequence (and/or amino acid analogues) defining at least one HCV epitope of either the E1 or the E2 region (see WO 96/04385).

**[0010]** It should also be understood that the isolates (biological samples) used in the examples section of the present invention were not intended to limit the scope of the invention and that any HCV isolate belonging to type 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or any other new genotype of HCV is a suitable source of HCV sequence for the practice of the present invention.

**[0011]** The HCV antigens used in the present invention may be full-length viral proteins, substantially full-length versions thereof, or functional fragments thereof (e.g. fragments which are not missing sequence essential to the formation or retention of an epitope). Furthermore, the HCV antigens of the present invention can also include other sequences that do not block or prevent the formation of any conformational epitope of interest. The presence or absence of a conformational epitope can be readily determined though screening the antigen of interest with an antibody (polyclonal serum or monoclonal antibody) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any). When in such screening polyclonal antibodies are used, it may be advantageous to adsorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest.

**[0012]** The term 'fusion polypeptide' intends a polypeptide in which the antigen(s), in particularly HCV antigen(s), are part of a single continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or be separated by spacer amino acid sequences. The fusion polypeptides may also contain amino acid sequences exogenous to HCV.

**[0013]** The term 'solid phase' or 'solid support' means a solid body to which the individual HCV antigens or the fusion polypeptide comprised of HCV antigens are bound covalently or by noncovalent means such as by hydrophobic adsorption. Examples of solid phases are microtiter plates, membrane strips such as nylon or nitrocellulose strips, and silicon chips.

**[0014]** The term 'biological sample' intends a fluid or tissue of a mammalian individual (e.g. an anthropoid, a human) that commonly contains antibodies produced by the individual, more particularly antibodies against HCV. The fluid or tissue may also contain HCV antigen. Such components are known in the art and include, without limitation, blood, plasma, serum, urine, spinal fluid, lymph fluid, secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk. white blood cells and myelomas. Body components include biological liquids. The term 'biological liquid' refers to a fluid obtained from an organism. Some biological fluids are used as a source of other products, such as clotting factors (e.g. Factor VIII), serum albumin, growth hormone and the like. In such cases, it is important that the source of biological fluid be free of contamination by virus such as HCV.

**[0015]** The term 'immunologically reactive' means that the antigen in question will react specifically with anti-HCV antibodies present in a body component from an HCV infected individual or an immunized individual.

**[0016]** The term 'immune complex' intends the combination formed when an antibody binds to an epitope on an antigen.

**[0017]** The terms E1 and E2 as used herein are fully described in WO 96/04385.

**[0018]** The term 'purified' as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35 % of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 85% , even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity as used herein. An 'isolated' HCV protein intends an HCV protein composition that is at least 35 % pure.

**[0019]** The term 'essentially purified proteins' refers to proteins purified such that they can be used for in vitro diagnostic methods and as a therapeutic compound. These proteins are substantially free from cellular proteins or DNA, vector-derived proteins or DNA, or other HCV viral components. Usually these proteins are purified to homogeneity (at least 80% pure, preferably, 85 % , more preferably, 90 % , more preferably 95 % , more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%, and most preferably the contaminating proteins should be undetectable by conventional methods like SDS-PAGE and silver staining.

**[0020]** The term 'recombinantly expressed' used within the context of the present invention refers to the fact that the proteins of the present invention are produced by recombinant expression methods be it in prokaryotes, or lower or higher eukaryotes as discussed in detail below.

**[0021]** The term 'lower eukaryote' refers to host cells such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular. Preferred lower eukaryotes are yeasts, particularly species within *Saccharomyces, Schizosaccharomyces, Klyveromyces, Pichia* (e.g. *Pichia pastoris*), *Hansenula* (e.g. *Hansenula polymorpha*), *Yarowia, Schwanniomyces, Zygosaccharonzyces* and the like. *Saccharomyces cerevisiae, S. carlsbergensis* and *K. lactis* are the most commonly used yeast hosts.

**[0022]** The term 'prokaryotes' refers to hosts such as *E.coli, Lactobacillus, Lactococcus, Salmonella, Streptococctcs, Bacillus subtilis* or *Streptomyces.* Also these hosts are contemplated within the present invention.

**[0023]** The term 'higher eukaryote' refers to host cells derived from higher animals, such as mammals, reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells), baby hamster kidney (BHK), pig kidney (PK15), rabbit kidney 13 cells (RK13), the human osteosarcoma cell line 143 B, the human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. *Spodoptera frugiperda*). The host cells may be provided in suspension or flask cultures, tissue cultures, organ cultures and the like. Alternatively the host cells may also be transgenic animals.

**[0024]** The term 'polypeptide' refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

**[0025]** The term 'recombinant polynucleotide or nucleic acid' intends a polynucleotide or nucleic acid of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

**[0026]** The term 'recombinant host cells', 'host cells', cells' , ' cell lines' , 'cell cultures', and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

**[0027]** The term 'replicon' is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

**[0028]** The term 'vector' is a replicon further comprising sequences providing replication and/or expression of a desired open reading frame.

**[0029]** The term 'control sequence' refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators: in eukaryotes, generally, such control sequences include promoters, and may include enhancers. The term 'control sequences' is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

**[0030]** The term 'promoter' is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent structural gene.

**[0031]** The expression 'operably linked' refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence 'operably linked' to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

**[0032]** An 'open reading frame' (ORF) is a region of a polynucleotide sequence which encodes a polypeptide and does not contain stop codons in the reading frame selected; this region may represent a portion of a coding sequence

or a total coding sequence.

**[0033]** A 'coding sequence' is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon. at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include but is not limited to mRNA. viral RNA, DNA (including cDNA), and recombinant polynucleotide sequences.

**[0034]** As used herein, 'epitope' or 'antigenic determinant' means an amino acid sequence that is immunoreactive. Generally an epitope consists of at least 3 to 4 amino acids, and more usually, consists of at least 5 or 6 amino acids, sometimes the epitope consists of about 7 to 8, or even about 10 amino acids. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof. Such equivalents also include strain, subtype (=genotype), or type(group)-specific variants, e.g. of the currently known sequences or strains belonging to genotypes 1a, 1b, 1c, 1d, 1e, 1f, 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 4h, 4i, 4j, 4k, 41, 5a, 5b, 6a, 6b, 6c, 7a, 7b, 7c, 8a, 8b, 9a, 9b, 10a, 11a, 12a or any other newly defined HCV (sub)type. It is to be understood that the amino acids constituting the epitope need not be part of a linear sequence, but may be interspersed by one or more series of any number of amino acids, thus forming a conformational epitope.

## AIMS OF THE INVENTION

**[0035]** It is an aim of the present invention to provide improved HCV diagnostic assay components and therapeutic proteins.

**[0036]** More particularly it is an aim of the present invention to provide improved HCV NS3 protein preparations for use in HCV antibody diagnosis.

**[0037]** It is further an aim of the present invention to provide a method for increasing the reactivity of HCV antibodies with recombinant or synthetic NS3 helicase protein or part thereof present on a solid phase.

**[0038]** It is also an aim of the present invention to provide new HCV NS3 protein encoding sequences.

**[0039]** It is also an aim of the present invention to provide new HCV NS3 protein encoding sequences of which the product does not react with falsly positive HCV samples.

**[0040]** It is another aim of the present invention to provide new HCV NS3 polypeptides.

**[0041]** It is another aim of the present invention to provide new HCV NS3 polypeptides which do not react with falsly positive HCV samples.

**[0042]** It is another aim of the present invention to provide a method for the detection of the polypeptides of the invention.

**[0043]** All the aims of the present invention are considered to have been met by the embodiments as set out below.

## SUMMARY OF THE INVENTION

**[0044]** The present invention relates to a solid phase immunoassay kit comprising on said solid phase an HCV NS3 protein or an analogue thereof, in the presence of a reducing agent, wherein said HCV NS3 protein or analogue thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 protease or helicase.

**[0045]** The present invention further relates to a immunoassay kit comprising a solid phase carrying, as antigen, an HCV NS3 protein or an analogue thereof, wherein said HCV NS3 protein or analogue thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 protease or helicase.

**[0046]** The present invention further relates to an immunoassay kit comprising a solid phase carrying as antigen an HCV NS3 protein or an analogue thereof, wherein said HCV NS3 protein or analogue thereof comprises and amino acid sequence defining at least one epitope of HCV NS3 protease or helicase, and wherein said kit which has been produced by adding a reducing agent in at least one of the steps below:

(i) coating of said solid phase with said antigen;
(ii) blocking said solid phase;
(iii) fixation of the proteins coated on said solid phase;
(iv) pretreatment of said solid phase.

**[0047]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is an HCV NS3 amino acid sequence selected from the group consisting of SEQ ID NO:3- 18.

**[0048]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is contained in a fusion protein.

**[0049]** The present invention further relates to an immunoassay kit as described above wherein said fusion protein

is selected from the group of amino acid sequences consisting of SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30 and SEQ ID NO:32.

**[0050]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is an HCV NS3 helicase protein or part thereof containing either S1200, A1218, A1384, P1407, V1412, P1424, or F1444, or a combination of one of said amino acids with any of the following amino acids selected from the group consisting of L1201, S1222, I1274, S1289, T1321, A1323, T1369, L1382, V1408, A1409, F1410, and wherein said HCV NS3 helicase protein or part thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 helicase.

**[0051]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is an HCV NS3 protein treated by a method comprising the steps of sulphonation and subsequent desulphonation.

**[0052]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is additionally treated with a zwitter-ionic detergent.

**[0053]** The present invention further relates to an immunoassay kit as described above wherein said HCV NS3 protein is treated with lauryldimethylbetaine as zwitter-ionic detergent.

**[0054]** The present invention further relates to a method for producing an immunoassay kit as described above wherein said reducing agent is present in at least one of the following steps:

(i) coating of said solid phase with said antigen; and
(ii) after (i), blocking said solid phase; and
(iii) after (ii), fixation of the proteins coated on said solid phase; and
(iv) after (iii), pretreatment of said solid phase.

**[0055]** The present invention further relates to a method as described above wherein said reducing agent is added in step (i).

**[0056]** The present invention further relates to a method as described above wherein said reducing agent is added in step (ii).

**[0057]** The present invention further relates to a method as described above wherein said reducing agent is added in steps (i) and (ii).

**[0058]** The present invention further relates to a method as described above wherein said reducing agent is added in step (iii).

**[0059]** The present invention further relates to a method as described above wherein said reducing agent is added in steps (i) and (iii).

**[0060]** The present invention further relates to a method as described above wherein said reducing agent is added in step (iv).

**[0061]** The present invention further relates to a method as described above wherein said reducing agent is added in steps (i) and (iv).

**[0062]** The present invention further relates to a method as described above wherein said reducing agent is DTT, DTE or TCEP.

**[0063]** The present invention further relates to a method as described above wherein said reducing agent is used in a concentration range of 0.1 mM to I M, more particularly from 0.5 mM to 500 mM, even more particularly from 1 mM to 250 mM, some applications may require ranges from 0.5 to 50 mM, 1 to 30 mM, 2 to 20 mM, or 5 to 15 mM, or about 10 mM.

**[0064]** The present invention further relates to an immunoassay kit as described above which is an ELISA kit, a QUICK test kit or a Line Immunoassay kit.

**[0065]** The present invention further relates to a method as described above wherein said produced immunoassay kit is an ELISA kit, a QUICK test kit or a Line Immunoassay kit.

**[0066]** The present invention further relates to a use of the immunoassay kit as described above for detecting antibodies to a HCV NS3 protein.

**[0067]** The present invention further relates to a use of the immunoassay kit as described above for detecting said antibodies in a biological sample.

**[0068]** The present invention further relates to a use of the immunoassay kit as described above for detecting early HCV NS3 seroconversion.

## DETAILED DESCRIPTION OF THE INVENTION

**[0069]** The present invention relates more particularly to a solid phase immunoassay comprising on said solid phase an HCV N53 antagen in the presence of a reducing agent. As is demonstrated in the Examples section the present

inventors have found that the presence of a reducing agent such as DTT, besides an antigen coated to a solid phase, renders a solid phase immunassay coupled antigen much more reactive with antibodies directed to said antigen. Also in solution, the antigen is rendered more reactive by reduction.

**[0070]** A reducing agent according to the present invention is any agent which achieves reduction of S-S disulfide bridges. Reduction of the 'S-S' disulfide bridges is a chemical reaction whereby the disulfides are reduced to thiol (-SH). The disulfide bridge breaking agents and methods are disclosed in WO 96/04385. S-S' Reduction can be obtained by (1) enzymatic cascade pathways or by (2) reducing compounds. Enzymes like thioredoxin, glutaredoxin are known to be involved in the in vivo reduction of disulfides and have also been shown to be effective in reducing 'S-S' bridges in vitro. Disulfide bonds are rapidly cleaved by reduced thioredoxin at pH 7.0, with an apparent second order rate that is around $10^4$ times larger than the corresponding rate constant for the reaction with DTT. The reduction kinetic can be dramatically increased by preincubation the protein solution with 1 mM DTT or dihydrolipoamide (Holmgren, 1979).

**[0071]** Thiol compounds able to reduce protein disulfide bridges are for instance Dithiothreitol (DTT), Dithioerythritol (DTE), β-mercaptoethanol, thiocarbamates, bis(2-mercaptoethyl) sulfone and N,N'-bis(mercaptoacetyl)hydrazine, and sodium-dithionite.

**[0072]** Reducing agents without thiol groups like ascorbate or stannous chloride ($SnCl_2$), which have been shown to be very useful in the reduction of disulfide bridges in monoclonal antibodies (Thakur et al., 1991), may also be used for the reduction of NS3. Sodium borohydride treatment has been shown to be effective for the reduction of disulfide bridges in peptides (Gailit, 1993). Tris (2-carboxyethyl)phosphine (TCEP) is able to reduce disulfides at low pH (Burns et al., 1991). Selenol catalyses the reduction of disulfide to thiols when DTT or sodium borohydride is used as reductant. Selenocysteamine. a commercially available diselenide, was used as precursor of the catalyst (Singh and Kats, 1995).

**[0073]** The present invention relates more particularly to a method for producing an immunoassay as defined above wherein said reducing agent is added to said solid phase during the steps of coating, blocking and/or fixation of said antigen to said solid phase.

**[0074]** The present invention also relates to a method for carrying out an immunoassay as defined above wherein said reducing agent is added during the step of pretreatment of the solid phase.

**[0075]** Coating conditions can vary widely as known by the skilled person and involves applying to a solid phase the protein and allowing a reaction to occur resulting in the binding of the protein to the solid phase. Binding can be, but is not restricted to, covalently hydrophobic or ionic bonds, Van Der Waels forces or hydrogen bridges. Different buffers known by the skilled man may be used for this step, including but not limited to carbamate and phosphate buffers.

**[0076]** Blocking can occur via any method known in the art and can for instance also be performed using albumin, serum proteins, polyvinylpyrolidone (PVP), detergents, gelatines. polyvinylalcohol (PVA) or caseine.

**[0077]** Fixation can occur according to any method known in the art.

**[0078]** Further examples of blocking, fixation and coating conditions are given in the Examples section.

**[0079]** The present invention relates even more particularly to a method as defined above wherein said reducing agent is added to said solid phase during the step of coating of the antigen to the solid phase. Examples of coating buffers are given in the Examples section. All other known coating buffers known in the art also form part of the present disclosure.

**[0080]** The present invention relates also to a method as defined above, wherein said reducing agent is added to said solid phase during the step of blocking said solid phase, comprising the antigen which had been applied thereto in the presence or absence of a reducing agent. Examples of blocking buffers are given in the Examples section. All other known blocking buffers known in the art also form part of the present disclosure.

**[0081]** The present invention relates also to a method as defined above, wherein said reducing agent is added to said solid phase during the step of fixation of the coated antigen to said solid phase comprising the antigen which had been applied thereto in the presence or absence of a reducing agent. The fixation step may also have been preceded by a blocking step in the presence or absence of a reducing agent. Examples of fixation buffers are given in the Examples section. All other known fixation buffers known in the art also form part of the present disclosure.

**[0082]** The present invention also relates to a method for carrying out an immunoassay as defined above wherein said reducing agent is added during the step of pretreatment of the solid phase before addition of the sample. Pretreatment of the plates can be done with plates that have been treated with a reducing agent in the coating, blocking and/or fixation step or with plates that have not been previously treated with a reducing agent.

**[0083]** Finally, the reducing agent may also be added during any further steps carried out in enzyme immunoassays, as part of the present invention, possibly after application of a reducing agent in one or more of the above 4 steps of coating, blocking, fixation and/or pretreatment. Such further steps include but are not limited to incubation the antibodies. detecting bound antibodies and color development.

**[0084]** The present invention relates preferably to a method as defined above wherein said reducing agent is DTT, DTE or TCEP.

**[0085]** The present invention relates also to a method as defined above wherein said reducing agent is used in a concentration range of 0.1 mM to 1 M, more particularly from 0.5 mM to 500 mM, even more particularly from 1 mM

to 250 mM, most partcularly from 1 to 50 mM. Some applications may require ranges from 0.5 to 50 mM, 1 to 30 mM, 2 to 20 mM, 5 to 15 mM, or about 10 mM reducing agent. Other applications require DTT concentrations of 50-500 mM, 100-300 mM or 200 mM. DTT is particularly preferred as a reducing agent.

**[0086]** The present invention relates to a method as defined above wherein said antigen is an HCV NS3 protein. More particularly an HCV NS3 helicase. Also any other protein known in the art may react better with antibodies against said protein when the protein is added to the solid phase in the presence of DTT, or treated with DTT thereafter.

**[0087]** The present invention also relates to a method as described above wherein said solid phase immunoassay comprises a combination of antigens of different aetiological agents or phenotypically linked conditions.

**[0088]** The present invention also relates to a solid phase immunoassay produced by a method as defined above. More particularly, a kit containing at least a solid phase such as a microtiterplate, a membrane strip or silicon chip which contains an antigen in the presence of a reducing agent.

**[0089]** More particularly, the present invention relates to an EUSA produced by a method as defined above.

**[0090]** In a preferred embodiment, the present invention relates to an ELISA produced by a method as defined above wherein said reducing agent is preferably added in the coating and/or fixation steps. In one preferred embodiment, the reducing agent can be applied in the coating step. In another preferred embodiment, the reducing agent can be applied in the fixation step. In a particularly preferred embodiment the reducing agent is added in both the coating and the fixation step.

**[0091]** In another preferred embodiment, the present invention relates to an EUSA produced by a method as defined above wherein said reducing agent is added during pretreatment of the plates before addition of the sample. Pretreatment of the plates can be done with plates that have been treated with a reducing agent in the coating and/or fixation step or with plates that have not been previously treated with a reducing agent. The reducing agent may also be added . during any further steps carried out in enzyme immunoassays. Such further steps include but are not limited to incubation the antibodies, detecting bound antibodies and color development.

**[0092]** The present invention also relates to an Line Immunoassay (LIA) produced by a method as defined above.

**[0093]** In a preferred embodiment, the present invention relates to a Line Immunoassay (LIA) produced by a method as defined above wherein said reducing agent is preferably added in the blocking step and/or washing step. The reducing agent may also be added during any further steps in producing or carrying out the enzyme immunoassays. Such further steps include but are not limited to fixation, pretreatment, incubation the antibodies, detecting bound antibodies and color development.

**[0094]** The present invention also relates to a QUICK assay produced by a method as defined above.

**[0095]** In a preferred embodiment, the present invention relates to a QUICK assay produced by a method as defined above wherein said reducing agent is preferably added during the coating of the antigen onto the strip. The QUICK assay is a lateral flow assay in which the antigens are coated onto the strips by spraying. In this assay, the reducing agent is preferably added to the spraysolution. The reducing agent may also be added during any further steps in producint or carrying out the enzyme immunoassays. Such further steps include but are not limited to blocking, fixation, pretreatment, incubation the antibodies, detecting bound antibodies and color development.

**[0096]** The present invention also relates to the use of an assay as defined above for in vitro diagnosis of antibodies raised against an antigen as defined above.

**[0097]** The present invention also relates to an immunoassay kit as defined above comprising HCV NS3 protein treated by a method comprising the steps of sulphonation and subsequent desulphonation.

**[0098]** Sulphonation and desulphonation is a reaction whereby $-SO_3$ groups are introduced or removed respectively from the protein.

**[0099]** Sulphonation is defined as a process where thiolgroups (SH) on proteins (R) and disulphide bonds are converted to S-Sulphonates, according to the following reactions:

$$RSH \text{ --- } > RS\text{-}SO_3^- \quad (1)$$

$$RS\text{-}SR + 2\ \text{-}SO_3^- + H_2O \text{ --- } > 2\ RS\text{-}SO_3^- + 2\ OH^- \quad (2)$$

**[0100]** The products of the reactions are S-Sulphoproteins which are usually stable at neutral pH. Reaction (1) can be obtained by incubation the protein sohuion with tetrathionate at pH>7 (Inglis and Liu, 1970). Reaction (2) proceeds to completion in the presence of copper ions (Cole, 1967). Chan (1968) has shown that treatment of protein with sodium sulfite and catalytic amounts of cysteine in the presence of oxygen gives sulpho-proteins.

**[0101]** Desulfonation can be obtained (1) by an excess of competitive -SH (thiol) groups, (2) by reducing agents or (3) by incubation in non-neutral pH conditions.

$$RS\text{-}SO_3^- + R'SH \text{ ---> } RSH + R'S\text{-}SO_3^-$$

$$RS\text{-}SO_3^- + \text{reducing agent ---> } RSH$$

**[0102]** Competitive thiol groups may be obtained from low molecular weight compounds or from proteinacous -SH groups.

**[0103]** Examples of mono- or dithiol containing compounds arc:

cysteine, cysteamine, reduced gluthation, N-acetyl cysteine, homocysteine, β-mercaptocthanol, thiocarbamates, bis(2-mercaptoethy])sulphone (BMS) and N,N'-bis(mercaptoacetyl)hydrazine (BMH), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Elman's reagent), Dithiotreitol (DTT) and Dithioerythrithiol (DTE).

**[0104]** The present invention further relates to an immunoassay kit as defined above comprising HCV NS3 protein as defined above which is additionally treated with a zwitterionic detergent. Empigen is known as lauryldimethylbetaine and is a particularly preferred example of a zwitterionic detergent. Other suitable detergents are known by the skilled man and are reviewed also in WO 96/04385.

**[0105]** The present invention further relates to a method for purifying a cysteine containing, recombinantly expressed protein, comprising at least 2, preferably 3 or 4, and even more preferably, all of the following steps:

(a) sulphonation of a lysate from recombinant host cells or lysis of recombinant host cells in the presence of guanidinium chloride (preferably 6 M Gu.HCl) and sulphonation of the cell lysate,
(b) treatment with a zwitterionic detergent, preferably after removal of the cell debris,
(c) purification of the sulphonated recombinant protein, or purification of the sulphonated recombinant pmtein with subsequent removal of the zwitterionic detergent, with said purification being preferably chromatography, more preferably a Ni-IMAC chromatography with said recombinant protein being a His-tagged recombinant protein.
(d) desulphonation of the sulphonated recombinant protein, preferably with a molar excess of a reducing agent such as DTT,
(e) storage in the presence of a molar excess of DTT.

Empigen is a particularly preferred example of a zwitterionic detergent. Inclusion of such a zwitterionic detergent and DTT was found to improve the purification protocol for HCV NS3 helicase and HCV envelope proteins.

**[0106]** The present invention also relates to the use of an HCV polynucleic acid encoding an HCV NS3 polyprotein as shown in Figure 1 (SEQ ID NOs 3-18) or a unique part of an HCV polynucleic acid having a sequence as represented in Figures 2-1. 3-1, 4-1, 5-1, 6-1, 7-1, and 8-1 (SEQ ID NOs 19, 21, 23, 25, 27, 29 and 31) for the preparation of an immunoassay kit as defined above.

**[0107]** The present invention also relates to an HCV polynucleic acid as defined above characterized in Figures 2-1, 3-1, 4-1, 5-1, 6-1, 7-1, and 8-1 and by the fact that its product does not react with false positive HCV samples, or a part thereof which encodes NS3 epitopes which do not react with false positive HCV samples. It was particularly surprising that the proteins coded by the clones represented by SEQ ID NOs 19, 21, 23, 25, 27, 29 and 31 have the property of not reacting with false positive HCV samples, yet they were able to react with most of the known NS3 antibody-positive samples after DTT treatement.

**[0108]** The present invention further relates to the use of a recombinant vector comprising a polynucleic acid as described.

**[0109]** The present invention further relates to the use of a host cell comprising a vector of the invention.

**[0110]** In addition to the reactivity gained by reduction, the NS3 reactivity is also severely determined by the sequence of the NS3 antigen.

**[0111]** The present invention therefore also relates to the use of an HCV polypeptide having part or all of the amino acid sequences as shown in Figures 1, 2-2, 3-2, 4-2, 5-2, 6-2, 7-2 and 8-2 (SEQ, ID NOs 20, 22, 24, 26, 28, 30, 32). The present invention also relates to the use of an HCV NS3 helicase protein as depicted in Figure 1 (SEQ ID NOs 1-18) or a unique part thereof.

**[0112]** The present invention also relates to the use of an HCV NS3 helicase protein or part thereof containing either S1200, A1218, A1384, P1407, V1412, P1424, or F1444, or a combination of these amino acids with any of the following amino acids L1201, S1222, 11274, S1289, T1321, A1323, T1369, L1382, V1408, A1409, or F1410. Said numbering is according to the commonly accepted HCV amino acid numbering system.

**[0113]** The present invention also relates to an immunoassay comprising an HCV NS3 polypeptide as defined above. Said immunoassay can be of any type of format known in the art (see for instance WO 96/13590 and Coligan et al.

1992). In particular, the present invention relates to a method for detecting a polypeptide of the invention comprising:

- contacting said polypeptide with a ligand binding to said polypeptide;
- determining the complex formed between said polypeptide and said ligand.

**[0114]** The term "a ligand" refers to any molecule able to bind the polypeptides of the present invention. The latter term specifically refers to polyclonal and/or monoclonal antibodies specifically raised (by any method known in the art) against the polypeptides of the present invention and also encompasses any antibody-like, and other, constructs as described in detail in EP 97870092.0 to Lorre et al. Such antibodies may be very useful for the detection of antigen in biological fluids. Detection of antigen can be done by any immunoassay known in the art such as assays which utilize biotin and avidin or streptavidin, ELISA's and immunoprecipitation, immunohistchemical techiques and agglutination assays. A detailed description of these assays is given in WO 96/13590.

**[0115]** The NS3 proteins of the present invention may also be used in any application where it is applicable to use an NS3 helicase, such as for drug screening purposes.

## FIGURE LEGENDS

**[0116]** **Figure 1.** Amino acid sequence of HCV NS3 clones isolated from HCV subtype 1a and 1b infected sera.

**[0117]** **Figure 2-1.** DNA coding sequence of the mTNFH6NS3 clone 19b fusion protein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0118]** **Figure 2-2.** Amino Acid sequence of the mTNFH6NS3 clone 19b fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence contains the mTNF fusion partner, the hexahistidine tag and part of the multilinker.

**[0119]** **Figure 3-1.** DNA coding sequence of the mTNFH6NS3 clone B9 fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusion partner, the hexahistidine tag and part of the multilinker.

**[0120]** **Figure 3-2.** Amino Acid sequence of the mTNFH6NS3 clone B9 fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusion partner, the hexahistidine tag and part of the multilinker.

**[0121]** **Figure 4-1.** DNA coding sequence of the mTNFH6NS3 Type 3a clone 21 fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0122]** **Figure 4-2.** Amino Acid sequence of the mTNFH6NS3 Type 3a clone 21 fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0123]** **Figure 5-1.** DNA coding sequence of the mTNFH6NS3 Type 3a clone 32 fusionprotein Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0124]** **Figure 5-2.** Amino Acid sequence of the mTNFH6NS3 Type 3a clone 32 fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0125]** **Figure 6-1.** DNA coding sequence of the mTNFH6NS3 Type 2a fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0126]** **Figure 6-2.** Amino Acid sequence of the mTNFH6NS3 Type 2a fusionprotein Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0127]** **Figure 7-1.** DNA coding sequence of the mTNFH6NS3 Type 2b fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0128]** **Figure 7-2.** Amino Acid sequence of the mTNFH6NS3 Type 2b fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0129]** **Figure 8-1.** DNA coding sequence of the mTNFH6NS3 Type 2c fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

**[0130]** **Figure 8-2.** Amino Acid sequence of the mTNFH6NS3 Type 2c fusionprotein. Sequence depicted in bold is non-NS3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

## EXAMPLES

### Example 1. Expression of HCV NS3 Type 1b clone 19b in *E. coli*

### 1.1 Cloning of the HCV NS3 Type 1b clones 19a and 19b genes

**[0131]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from HCV subtype 1b serum

IG8309 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers HCPr59 (5'-GGGCCCCACCAT-GGGGGTTGCGAAGGCGGTGGACTT-3') (SEQ ID NO 1) and HCPr60 (5'-CTATTAGCTGAAAGTCGACTGTCTGGGT-GACAGCA-3') (SEQ ID NO 2). This yielded a PCR fragment 19 which was cloned into *E. coli* The sense primer HCPr59 introduces an ApaI restriction site which includes an artifical methionine. Antisense oligonucleotide HCPr60 introduces a stopcodon after aa 1465. The PCR fragment was subsequently cut with ApaI and the resulting 833 bp ApaI fragment was cloned in the ApaI-cut expressionvector pmTNFHRP (Innogenetics, Ghent, Belgium). Four hepatitis C clones (HCCI) were sequenced HCC119a and HCC119b (see deduced amino acid sequence given in Figure I and Figure 2-2). Clone HCC119b (pmTiNTHRPHCC119b) was retained for further subcloning.

**1.2 Construction of the expression plasmid pEmTNFMPHHCC119b**

**[0132]** Starting from vector pmTNFHRPHCC119b the NS3 clone 19b coding sequence was isolated as a 900 bp NcoI fragment and inserted into the NcoI-cut expressionvector pEmTNFMPH (Innogenetics, Ghent, Belgium) resulting in vector pEmTNFNFPHHCC119b. This plasmid expresses HCV NS3 clone 19b as an N-terminal fusionprotein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs 19 and 20; Figure 2).

**1.3 Expression of HCV NS3 clone 19b in *E.coli***

**[0133]** *E,. coli* strain MC1061(pAcI) cells (Wertman et al., 1986) were transformed with plasmid pEmTNFMPHHCC119b. MC 1061 (pacI) cells harboring pEmTNFMPHHCC119b were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 23°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an $OD_{600}$ of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 clone 19b fusion protein was analysed by western blotting using specific monoclonal antibodies and HCV positive human sera.

**Example 2. Expression of HCV NS3 clone B9 in *E.coli***

**2.1 Cloning of the HCV NS3 Type 1a clone B9 gene**

**[0134]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from HCV subtype Ia serum IG21054 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers HCPr59 (5'-GGGCCCCACCAT-GGGGGTTGCGAAGGCGGTGGACTT-3') (SEQ ID NO 1) and HCPr60 (5'-CTATTAGCTGAAAGTCGACTGTCTGGGT-GACAGCA-3') (SEQ ID NO 2). This yielded a PCR fragment B which was cloned into *E. coli.* The sense primer HCPr59 introduces an ApaI restriction site which includes an artifical methionine. Antisense oligonucleotide HCPr60 introduces a stopcodon after aa 1465. The PCR fragment was subsequently cloned in the pGEM-T vector (Promega, Madison, WI, US). Four clones were sequenced: B7, B9, B12, and B 14 (see deduced amino acid sequences in Figure 1 and Figure 3-2). Clone B9 (pGEMTNS3B9) was retained for further subcloning.

**2.2 Construction of the expression plasmid pIGFH111NS3B9**

**[0135]** Starting from vector pGEMTNS3B9, the clone B9 coding sequence was isolated as a 850 bp NcoI/SpeI blunted fragment and inserted into the NcoI/StuI cut expression vector pIGFH111 (Innogenetics, Ghent, Belgium) resulting in vector pIGFH111NS3B9. This plasmid expresses HCV NS3 clone B9 as an N-terminal fusion protein with the N-terminal 25 aa of murine TNT followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs. 21 and 22; Figure 3).

**2.3 Expression of HCV NS3 clone B9 in *E.coli***

**[0136]** *E. coli* strain MC106 (pAcI) (Wertman et al., 1986) cells were transformed with plasmid pIGFH111NS3B9. MC1061(pAcI) cells harboring pIGFH111NS3B9 were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an $OD_{600}$ of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 clone B9 fusion protein was analysed on Western blot using specific monoclonal antibodies and HCV positive human sera.

**Example 3. Expression of HCV NS3 Type 1a clones A26, C16., and D18 in *E.coli***

**[0137]** Clones A26, C16, and D18 were isolated from HCV subtype I a infected sera IG21051, IG17790, and IG21068, respectively, in a similar way as described for clone B9 using primers HCPr59 and HCPr60. Initially, clones, A5, A26, C1, C3, C4, C12, C16, D17, D18, and D19, were cloned and sequenced (see deduced amino acid sequences given in Figure 1). Clones A26, C16, and D18 were retained for further subcloning

**Example 4. Expression of HCV NS3 Type 3a clones 21 and 32 in *E.coli***

**4.1 Cloning of the HCV NS3 Type 3a clones 21 and 32 genes**

**[0138]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from HCV subtype 3a sera IG21349 and IG20014 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers 403 (5'GGGCCCCAC-CATAGGTGTAGCAAAAGCCCTACAGTT-3') (SEQ ID NO 33) and 404 (5'-CTATTAGCTGAAGTCAACGTACTGT-TCAACAGC-3') (SEQ ID NO 34). This yielded in both cases a PCR fragment of approx. 850 bp which was subsequently subcloned in the pGEM-T vector (Promega, Madison, WI, US). From each cloned PCR fragment several clones were sequenced but from each serum only one cloned fragment proved to be completely correct upon sequencing. This was clone 21 (pGEM-TNS3T3a.21) for serum IG21349 and clone 32 (pGEM-TNS3T3a.32) for serum IG20014 (Figures 4 and 5).

**4.2 Construction of the expressionplasmids pIGFH111NS3T3a.21 and pIGFH111NS3T3a.32**

**[0139]** Starting from vectors pGEM-TNS3T3a.21 and pGEM-TNS3T3a.32, the clone 21 and 32 coding sequences were isolated as 850 bp NcoI/SalI fragments and inserted into the NcoI/SalI cut expression vector pIGFH111 (Innogenetics, Ghent, Belgium) resulting in vectors pIGFH111NS3T3a.21 and pIGFH111NS3T3a.32, respectively, These plasmids express HCV NS3 Type 3a clones 21 and 32 as N-terminal fusion proteins with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs 23-26; Figures 4 and 5).

**4.3 Expression of HCV NS-3 Type 3a clones 21 and 32 in *E.coli***

**[0140]** *E.coli* strain MC106I(pAcI) (Wertman et al., 1986) cells were transformed with plasmids pIGFH111NS3T3a.21 and pIGFH111NS3T3a.32, respectively. MC1061(pAcI) cells harboring pIGFH111NS3T3a.21 or pIGFH111NS3T3a.32 were grown overnight in Luria Broth (LB) supplemented with 10 μg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD600 of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 Type 3a clones 21 and 32 fusionproteins was analysed on Western blot using specific monoclonal antibodies and HCV positive human sera.

**Example 5. Expression of HCV NS3 Type 2a clone 3 in *E.coli***

**5.1 Cloning of the HCV NS3 Type 2a clone 3 gene**

**[0141]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from a HCV subtype 2a serum IG21342 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers 412 (6'-GGGCCCCACCATGGGCGT-GGCCAAGTCCATAGACTT-3') (SEQ ID NO 35) and 413 (5'-CTATTAGCTGAAGTCTACAACTTGAGTGACCGC-3') (SEQ ID NO 36). This yields a PCR fragment of approx. 850 bp which was subsequently subcloned in the pGEM-T vector (Promega, Madison, WI, US). Several clones were sequenced and clone 3 (pGEM-TNS3T2a) was retained for further subcloning (Figure 6).

**5.2 Construction of expressionplasmid pIGFH111NS3T2a**

**[0142]** Starting from vector pGEM-TNS3T2a, the clone 3 coding sequence was isolated as a 850 bp NcoI fragment and inserted into the NcoI cut expression vector pIGFH111 (Innogenetics Ghent, Belgium) resulting in vector pIGFH111NS3T2a. This plasmid expresses HCV NS3 Type 2a clone 3 as an N-terminal fusion protein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs 27 and 28; Figure 6).

### 5.3 Expression of HCV NS-3 Type 2a clone 3 in *E.coli*

**[0143]** *E.coli* strain MC1061(pAcI) (Wertman et al., 1986) cells were transformed with plasmid pIGFH111NS3T2a. MC1061(pAcI) cells harbouring pIGFH111NS3T2a were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD600 of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 Type 2a clone 3 fusionprotein was analysed on Western blot using specific monoclonal antibodies and HCV positive human sera.

## Example 6. Expression of HCV NS3 Type 2b clone 9 in *E.coli*

### 6.1 Cloning of the HCV NS3 Type 2b clone 9 gene

**[0144]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from a HCV subtype 2b serum IG20192 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers 401 (5'-GGGCCCCACCATGGGCG-TAGCCAAATCCATTGACTT-3') (SEQ ID NO 37) and 402 (5'-CTATTAGCTGAAGTCTACAATTTGAGAGACCGC-3') (SEQ ID NO 38). This yields a PCR fragment of approx. 850 bp which was subsequently subcloned in the pGEM-T vector (Promega, Madison, WI, US). Several clones were sequenced and clone 9 was retained for further subcloning (Figure 7).

### 6.2 Construction of expressionplasmid pIGFH111NS3T2b

**[0145]** Starting from vector pGEM-TNS3T2b, the clone 9 coding sequence was isolated as a 850 bp NcoI fragment and inserted into the NcoI cut expression vector pIGFH11 (Innogenetics, Ghent, Belgium) resulting in vector pIGFH111NS3T2b. This plasmid expresses HCV NS3 Type 2b clone 9 as an N-terminal fusion protein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs 29-30; Figure 7)

### 6.3 Expression of HCV NS-3 Type 2b clone 9 in *E.coli*

**[0146]** *E. coli* strain MC 1061 (pAcI) cells (Wertman et al., 1986) were transformed with plasmid pIGFH111NS3T2b. MCI061(pAcI) cells harbouring pIGFH111NS3T2b were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD600 of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 Type 2b clone 9 fusionprotein was analysed on Western blot using specific monoclonal antibodies and HCV positive human sera.

## Example 7. Expression of HCV NS3 Type 2c clone 14 in *E.coli*

### 7.1 Cloning of the HCV NS3 Type 2c clone 14 gene

**[0147]** The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from a HCV subtype 2c serum IG20031 (Innogenetics, Ghent, Belgium) using synthetic oligonucleotide primers 401 (5'-GGGCCCCACCATGGGCG-TAGCCAAATCCATTGACTT-3') (SEQ ID NO 37) and 402 (5'-CTATTAGCTGAAGTCTACAATTTGAGAGACCGC-3') (SEQ ID NO 38). This yields a PCR fragment of approx. 850 bp which was subsequently subcloned in the pGEM-T vector (Promega, Madison, WI, US). Several clones were sequenced and clone 14 (pGEM-TNS3T2c) was retained for further subcloning (Figure 8).

### 7.2 Construction of expressionplasmid pIGFH111NS3T2c

**[0148]** Starting from vector pGEM-TNS3T2c, the clone 14 coding sequence was isolated as a 850 bp NcoI fragment and inserted into the NcoI cut expression vector pIGFH111 (Innogenetics, Ghent, Belgium) resulting in vector pIGFH111NS3T2c. This plasmid expresses HCV NS3 Type 2c clone 14 as an N-terminal fusion protein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site (SEQ ID NOs 31 and 32; Figure 8)

### 7.3 Expression of HCV NS-3 Type 2c clone 14 in *E.coli*

**[0149]** E.coli strain MC1061(pAcI) cells (Wertman et al., 1986) were transformed with plasmid pIGFH111NS3T2c. MC1061(pAcI) cells harbouring pIGFHI 11NS3T2c were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD600 of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 Type 2c clone 14 fusionprotein was analysed on Western blot using specific monoclonal antibodies and HCV positive human sera.

### Example 8. Purification of the NS3 helicase protein domain

**[0150]** Nine volumes of 8M Guanidinium hydrochloride (Gu.HCl) and 1 volume of 0.2 M $NaHPO_4$ were added to each gram equivalent of wet *E. coli* cell paste and the solution was homogenized by continuously vortexing. Solid $Na_2S_4O_6$ and $Na_2SO_3$ were added to the solution up to a final concentration of 65 and 360 mM, respectively. $CuSO_4$ (stock solution: 0.1 M in 25% $NH_3$) was added up to a final concentration of 100µM. The solution was stirred overnight in the dark at room temperature and after incubation at -70°C cleared by centrifugation at 4°C (30 min, 20.000 rpm, JA20 rotor).

Lauryldimethylbetaine (also known as Empigen BB®; Albright & Wilson Ltd., Oldbuly, UK) and imidazole were added to the supernatant up to a final concentration of 1% (w/v) and 20 mM, respectively. The pH was adjusted to 7.2 with IN HCl. A sample corresponding to 3 L cell culture equivalent was loaded at 2 mL/min on a 25 mL Ni-IDA Sepharose FF (XK 16/20 column, Pharmacia, Upsala, Sweden), which had been equilibrated with buffer A containing 20 mM imidazole (buffer A: 50 mM phosphate, 6M Gu.HCl, 1% Lauryldimethylbetaine pH 7.2). The Ni-IDA Sepharose column was washed consecutively with:

- buffer A containing 20mM imidazole
- buffer A containiag 35 mM imidazole
- buffer A containing 50 mM imidazole
- buffer B containing 50 mM imidazole (buffer B: 50 mM phosphate, 6M Gu.HCl, pH 7.2)
- buffer B containing 200 mM imidazole.

Each washing step was maintained during the chromatography untill the absorbance at 280 nm reached baseline level. The column was regenerated with 50 mM EDTA, 500 mM NaCl, pH 7.0.

**[0151]** fractions were analysed by SDS-PAGE using non-reduang conditions and silver staining. The mTNF-NS3 B9 fusion protein was recovered in the 200 mM imidazole elution. Western blotting using rabbit anti-human TNF (1µg N53/lane) and rabbit anti-*E coli* (10 µg NS3/lane) showed that the NS3 exhibited a purity of over 99 % after this single chromatography step.

**[0152]** The 200 mM imidazole elution fractions were pooled and desalted.

**[0153]** A 40 mL Ni-IDA eluate sample was loaded at 10 mL /min on a 300 mL Sephadex G25 column (XK 50, Pharmacia, Upsala, Sweden) which had been equilibrated with 50 mM phosphate, 6M ureum, 1mM EDTA, pH 7.2. 10 mL-fractioas were collected and the protein concentration was determined by the micro BCA method (Pierce, Rockford, IL, US). The protein concentration was adjusted to 500 µg/mL, with the desalting buffer before desulphonation and reduction. The overall yield was 50-55 mg purified NS3 fusion protein/L culture equivalent.

**[0154]** Finally, DTT (stock solution: 100 mM in destilled water) was added in a 100-fold molar excess versus the cysteine content in the NS3 antigen (e.g. NS3 19b contains 7 cysteins). The solution was flushed with nitrogen and incubated for 1h at 28°C. The NS3 sample was subsequently diluted in the appropiate buffer for ELISA and LIA coating.

### Example 9. NS3 helicase antibody reactivity tested in LIA

**[0155]** In order to test the NS3 helicase antibody reactivity, a line of 50 µg/ml NS3 antigen solution in phosphate buffered saline was applied onto nylon membrane strips. The strips were dried for at least 1 hour at a temperature between 18-24°C and were subsequently blocked with PBS/caseine in the presence (10 mM) or absence of the reducing agent DTT. The strips were subsequently washed with PBS containing Tween 20 and either no DTT or 10 mM DTT and with water containing either no DTT or 10 mM DTT and 1 mM EDTA. The membranes were dried for 30 minutes and cut into strips for testing of different patient samples.

**[0156]** The results of an experiment wherein strips were incubated with the anti-HCV seroconversion panel PHV903 (Boston Biomedica Inc., Boston, US) are given in Table 1.

**Example 10. NS3 helicase antibody reactivity tested in ELISA**

**[0157]** In order to test the NS3 helicase antibody reactivity, ELISA plates were coated with the NS3 antigens purified as in Example 8 in the following way.

**[0158]** Microtiter plate wells were coated with NS3 protein at a concentration of 0.3 µg/ml NS3 protein in coating buffer containing 50 mM carbonate buffer, either 200 mM DTT or no DTT, and 1 mM EDTA. The microtiter plates are incubated for 18 hours at 20° C, and blocked with 300 µl of PBS/caseine buffer per well. The plates were incubated for 2 hours at 20°C and subsequently fixed with 300 µl of fixation buffer containing either 200 mM DTT or no DTT, and 1 mM EDTA for 2 hours at 20°C.

**[0159]** The results are shown in Tables 2 and 3. Table 2 gives the Signal to Noise values of assays including NS3 coated and fixed with or without DTT, with the BBI seroconversion panels PHV901 to PHV912. Table 3 shows a summary of the number of days in which HCV antibodies can be detected earlier by the assay incorporating DTT. Clearly, a total number of 34 days of earlier detection in 12 HCV seroconversions can be obtained by incorporating DTT in the assay.

Table 1.

| **BBI panels tested in LIA coated with HCV NS3 as described in example 9.** | | |
|---|---|---|
| **PHV** | **+DTT[1]** | **-DTT[1]** |
| 903-01 | - | - |
| 903-02 | - | - |
| 903-03 | +/- | - |
| 903-04 | 2 | - |
| 903-05 | 2 | +/- |
| 903-06 | 2 | +/- |
| 903-07 | 4 | 2 |
| 903-08 | 4 | 2 |

[1]-: no reaction; +positive reaction; intensity ratings are given in comparison with different cut off lines sprayed onto the same strip.

Table 2:

| **BBI panels-tested in ELISA coated with HCV NS3 as described in example 10.** | | | |
|---|---|---|---|
| **MEMBER ID#** | **BLEED DATE** | **+ DTT ($OD_{150}$)** | **- DTT ($OD_{150}$)** |
| PHV901-01 | 09/23/93 | 0.1 | 0.3 |
| PHV901-02 | 11/27/93 | 0.1 | 0.3 |
| PHV901-03 | 12/29/93 | 2.0 | 2.9 |
| PHV901-04 | 12/31/93 | 2.1 | 3.0 |
| PHV901-05 | 01/05/94 | 2.2 | 3.1 |
| PHV901-06 | 01/07/94 | 2.4 | 3.2 |
| PHV901-07 | 02/01/94 | 4.1 | 6.0 |
| PHV901-08 | 02/09/94 | 3.9 | 5.9 |
| PHV901-09 | 03/01/94 | 4.0 | 7.9 |
| PHV901-10 | 03/08/94 | 4.1 | 7.8 |
| PHV901-11 | 04/14/94 | 4.2 | 8.3 |
| | | | |
| PHV903-01 | 02/07/92 | 0.2 | 0.2 |
| PHV903-02 | 02/12/92 | 0.9 | 0.9 |

Table 2: (continued)

| BBI panels-tested in ELISA coated with HCV NS3 as described in example 10. | | | |
|---|---|---|---|
| MEMBER ID# | BLEED DATE | + DTT ($OD_{150}$) | - DTT ($OD_{150}$) |
| PHV903-03 | 02/14/92 | 1.3 | 1.6 |
| PHV903-04 | 02/19/92 | 2.5 | 2.7 |
| PHV903-05 | 02/21/92 | 2.8 | 2.8 |
| PHV903-06 | 02/26/92 | 3.2 | 4.6 |
| PHV903-07 | 02/28/92 | 3.5 | 5.4 |
| PHV903-08 | 03/04/92 | 3.5 | 4.1 |
| | | | |
| PHV904-01 | 04/18/95 | 0.1 | 0.2 |
| PHV904-02 | 04/20/95 | 0.1 | 0.3 |
| PHV904-03 | 04/25/95 | 0.1 | 0.2 |
| PHV904-04 | 04/27/95 | 0.1 | 0.2 |
| PHV904-05 | 05/02/95 | 0.4 | 0.4 |
| PHV904-06 | 05/09/95 | 0.8 | 0.5 |
| PHV904-07 | 05/11/95 | 0.8 | 0.5 |
| | | | |
| PHV905-01 | 11/17/95 | 0.1 | 0.2 |
| PHV905-02 | 11/21/95 | 0.1 | 0.3 |
| PHV905-03 | 11/24/95 | 0.1 | 0.3 |
| PHV905-04 | 11/28/95 | 0.2 | 0.3 |
| PHV905-05 | 12/01/95 | 0.5 | 0.3 |
| PHV905-06 | 12/05/95 | 1.0 | 0.4 |
| PHV905-07 | 12/08/95 | 2.5 | 0.8 |
| PHV905-08 | 12/12/95 | 3.5 | 2.2 |
| PHV905-09 | 12/15/95 | 3.5 | 3.2 |
| PHV 907-01 | 04/06/96 | 0.1 | 0.2 |
| PHV907-02 | 04/10/96 | 0.1 | 0.2 |
| PHV907-03 | 04/13/96 | 0.1 | 0.2 |
| PHV907-04 | 04/19/96 | 3.0 | 2.2 |
| PHV907-05 | 04/24/96 | 3.7 | 4.1 |
| PHV907-06 | 04/27/96 | 3.6 | 4.1 |
| PHV907-07 | 09/17/96 | 3.9 | 7.6 |
| | | | |
| PHV908-01 | 01/26/96 | 0.1 | 0.1 |
| PHV908-02 | 01/29/96 | 0.1 | 0.1 |
| PHV908-03 | 01/31/96 | 0.1 | 0.1 |
| PHV908-04 | 02/06/96 | 0.1 | 0.1 |
| PHV908-05 | 02/08/96 | 0.1 | 0.1 |

Table 2: (continued)

| BBI panels-tested in ELISA coated with HCV NS3 as described in example 10. | | | |
|---|---|---|---|
| **MEMBER ID#** | **BLEED DATE** | **+ DTT ($OD_{150}$)** | **- DTT ($OD_{150}$)** |
| PHV908-06 | 02/14/96 | 0.2 | 0.1 |
| PHV908-07 | 02/20/96 | 1.4 | 0.2 |
| PHV908-08 | 02/22/96 | 1.6 | 0.2 |
| PHV908-09 | 02/27/96 | 1.9 | 0.2 |
| PHV908-10 | 03/01/96 | 2.3 | 0.2 |
| PHV908-11 | 03/07/96 | 2.3 | 0.4 |
| PHV908-12 | 03/11/96 | 2.8 | 0.5 |
| PHV908-13 | 03/14/96 | 2.8 | 0.5 |
| | | | |
| PHV909-01 | 01/28/96 | 0.1 | 0.4 |
| PHV909-02 | 02/15/96 | 2.3 | 5.4 |
| PHV909-03 | 02/17/96 | 2.4 | 5.3 |
| | | | |
| PHV910-01 | 08/26/96 | 0.1 | 0.2 |
| PHV910-02 | 08/30/96 | 0.4 | 0.2 |
| PHV910-03 | 09/03/96 | 2.7 | 3.1 |
| PHV910-04 | 09/06/96 | 3.6 | 6.4 |
| PHV910-05 | 09/10/96 | 3.9 | 8.1 |
| | | | |
| PHV911-01 | 10/30/96 | 0.1 | 0.2 |
| PHV911-02 | 11/02/96 | 0.1 | 0.2 |
| PHV911-03 | 11/13/96 | 2.1 | 4.0 |
| PHV911-04 | 11/20/96 | 3.6 | 7.8 |
| PHV911-05 | 11/23/96 | 3.7 | 7.7 |
| | | | |
| PHV912-01 | 01/06/96 | 0.2 | 0.3 |
| PHV912-02 | 01/10/96 | 0.2 | 0.2 |
| PHV912-03 | 01/13/96 | 4.5 | 9.9 |
| PHV902-01 | 02/10/92 | 0.1 | 0.2 |
| PHV902-02 | 02/12/92 | 0.1 | 0.2 |
| PHV902-03 | 02/17/92 | 0.1 | 0.3 |
| PHV902-04 | 02/19/92 | 0.3 | 0.6 |
| PHV902-05 | 02/24/92 | 2.6 | 3.9 |
| PHV902-06 | 02/26/92 | 3.1 | 5.9 |
| PHV902-07 | 03/02/92 | 3.4 | 6.5 |
| | | | |
| PHV906-01 | 10/07/95 | 0.5 | 0.3 |

Table 2: (continued)

| BBI panels-tested in ELISA coated with HCV NS3 as described in example 10. | | | |
|---|---|---|---|
| MEMBER ID# | BLEED DATE | + DTT ($OD_{150}$) | - DTT ($OD_{150}$) |
| PHV906-02 | 10/09/95 | 0.5 | 0.4 |
| PHV906-03 | 10/14/95 | 1.6 | 0.6 |
| PHV906-04 | 10/17/95 | 1.5 | 1.2 |
| PHV906-05 | 10/21/95 | 2.2 | 3.0 |
| PHV906-06 | 10/24/95 | 2.5 | 4.5 |
| PHV906-07 | 10/28/95 | 2.9 | 5.7 |

Table 3.

| Overview of the BBI panels - numbers of days with earlier detection | | |
|---|---|---|
| PHV | +DTT | -DTT |
| 901 | 0 | 0 |
| 902 | 0 | 0 |
| 903 | 0 | 0 |
| 904 | 0 | 0 |
| 905 | 7 | 0 |
| 906 | 3 | 0 |
| 907 | 0 | 0 |
| 908 | 24 | 0 |
| 910 | 0 | 0 |
| 911 | 0 | 0 |
| 912 | 0 | 0 |

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## Claims

1. A solid phase immunoassay kit comprising on said solid phase an HCV NS3 protein, or an analogue thereof, in the presence of a reducing agent, wherein said HCV NS3 protein or analogue thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 protease or helicase.

2. An immunoassay kit comprising a solid phase carrying, as antigen, an HCV NS3 protein or an analogue thereof which has been reduced by a reducing agent, wherein said HCV NS3 protein or analogue thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 protease or helicase.

3. An immunoassay kit comprising a solid phase carrying as antigen an HCV NS3 protein or an analogue thereof, wherein said HCV NS3 protein or analogue thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 protease or helicase, and wherein said kit which has been produced by adding a reducing agent in at least one of the steps below:

    (i) coating of said solid phase with said antigen;
    (ii) blocking said solid phase;
    (iii) fixation of the proteins coated on said solid phase;
    (iv) pretreatment of said solid phase.

4. The immunoassay kit according to any of claims 1 to 3 wherein said HCV NS3 protein is an HCV NS3 amino acid sequence selected from the group consisting of SEQ ID NO:3- 18.

5. The immunoassay kit according to any of claims 1 to 4 wherein said HCV NS3 protein is contained in a fusion protein.

6. The immunoassay kit according to claim 5 wherein said fusion protein is selected from the group of amino acid sequences consisting of SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30 and SEQ ID NO:32.

7. The immunoassay kit according to any of claims 1 to 6 wherein said HCV NS3 protein is an HCV NS3 helicase protein or part thereof containing either S1200, A1218, A1384, P1407, V1412, P1424, or F1444, or a combination of one of said amino acids with any of the following amino acids selected from the group consisting of L1201, S1222, I1274, S1289, T1321. A1323, T1369, L1382, V1408, A1409, F1410, and wherein said HCV NS3 helicase protein or part thereof comprises an amino acid sequence defining at least one epitope of HCV NS3 helicase.

8. The immunoassay kit according to any of claims 1 to 7 wherein said HCV NS3 protein is an HCV NS3 protein treated by a method comprising the steps of sulphonation and subsequent desulphonation.

9. The immunoassay kit according to claim 8 wherein said HCV NS3 protein is additionally treated with a zwitter-ionic detergent.

10. The immunoassay kit according to claim 9 wherein said HCV NS3 protein is treated with lauryldimethylbetaine as zwitter-ionic detergent.

11. A method for producing an immunoassay kit according to any of claims 1 to 10 wherein said reducing agent is present in at least one of the following steps:

    (i) coating of said solid phase with said antigen; and
    (ii) after (i), blocking said solid phase; and
    (iii) after (ii), fixation of the proteins coated on said solid phase; and
    (iv) after (iii), pretreatment of said solid phase.

12. The method according to claim 11 wherein said reducing agent is added in step (i).

13. The method according to claim 11 wherein said reducing agent is added in step (ii).

14. The method according to claim 11 wherein said reducing agent is added in steps (i) and (ii).

15. The method according to claim 11 wherein said reducing agent is added in step (iii).

16. The method according to claim 11 wherein said reducing agent is added in steps (i) and (iii).

17. The method according to claim 11 wherein said reducing agent is added in step (iv).

18. The method according to claim 11 wherein said reducing agent is added in steps (i) and (iv).

19. Method according to any of claims 11 to 18 wherein said reducing agent is DTT, DTE or TCEP.

20. Method according to any of claims 11 to 19 wherein said reducing agent is used in a concentration range of 0.1 mM to 1 M, more particularly from 0.5 mM to 500 mM, even more particularly from 1 mM to 250 mM, some applications may require ranges from 0.5 to 50 mM, 1 to 30 mM, 2 to 20 mM, or 5 to 15 mM, or about 10 mM.

21. The immunoassay kit according to any of claims 1 to 3 which is an ELISA kit, a QUICK test kit or a Line Immunoassay kit.

22. The method according to claim 11 wherein said produced immunoassay kit is an ELISA kit, a QUICK test kit or a Line Immunoassay kit.

23. Use of the immunoassay kit according to any of claims 1 to 3 for detecting antibodies to a HCV NS3 protein.

24. Use of the immunoassay kit according to claim 23 for detecting said antibodies in a biological sample.

25. Use of the immunoassay kit according to any of claims 1 to 3 for detecting early HCV NS3 seroconversion.

**Patentansprüche**

1. Festphasen-Immunassay-Kit mit einem auf der Festphase in Gegenwart eines Reduktionsmittels vorhandenen HCV-NS3-Protein oder einem Analogon davon, wobei das HCV-NS3-Protein oder Analogon davon eine Aminosäuresequenz umfaßt, durch die wenigstens ein Epitop der HCV-NS3-Protease oder -Helicase definiert ist.

2. Immunassay-Kit mit einer Festphase, die ein HCV-NS3-Protein oder ein Analogon davon, das durch ein Reduktionsmittel reduziert wurde, als Antigen trägt, wobei das HCV-NS3-Protein oder Analogon davon eine Aminosäuresequenz umfaßt, durch die wenigstens ein Epitop der HCV-NS3-Protease oder -Helicase definiert ist.

3. Immunassay-Kit mit einer Festphase, die ein HCV-NS3-Protein oder ein Analogon davon als Antigen trägt, wobei das HCV-NS3-Protein oder Analogon davon eine Aminosäuresequenz umfaßt, durch die wenigstens ein Epitop der HCV-NS3-Protease oder -Helicase definiert ist und wobei der Kit durch Zugabe eines Reduktionsmittels in wenigstens einem der unten aufgeführten Schritte hergestellt wurde:

   (i) Beschichten der Festphase mit dem Antigen;
   (ii) Blockieren der Festphase;
   (iii) Fixieren der auf der Festphase aufgebrachten Proteine;
   (iv) Vorbehandeln der Festphase.

4. Immunassay-Kit nach einem der Ansprüche 1 bis 3, wobei es sich bei dem HCV-NS3-Protein um eine HCV-NS3-Aminosäuresequenz, ausgewählt aus der Gruppe bestehend aus SEQ ID NO:3-18, handelt.

5. Immunassay-Kit nach einem der Ansprüche 1 bis 4, wobei das HCV-NS3-Protein in einem Fusionsprotein enthalten ist.

6. Immunassay-Kit nach Anspruch 5, wobei das Fusionsprotein ausgewählt ist aus der Gruppe von Aminosäuresequenzen, bestehend aus SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30 und SEQ ID NO:32.

7. Immunassay-Kit nach einem der Ansprüche 1 bis 6, wobei es sich bei dem HCV-NS3-Protein um ein HCV-NS3-Helicaseprotein oder einen Teil davon mit entweder S1200, A1218, A1384, P1407, V1412, P1424 oder F1444 oder einer Kombination einer dieser Aminosäuren mit einer der folgenden Aminosäuren, ausgewählt aus der Gruppe bestehend aus L1201, S1222, I1274, S1289, T1321, A1323, T1369, L1382, V1408, A1409, F1410, handelt und wobei das HCV-NS3-Helicaseprotein oder ein Teil davon eine Aminosäuresequenz umfaßt, durch die wenigstens ein Epitop der HCV-NS3-Helicase definiert ist.

8. Immunassay-Kit nach einem der Ansprüche 1 bis 7, wobei es sich bei dem HCV-NS3-Protein um ein HCV-NS3-Protein handelt, das nach einem die Schritte Sulfonierung und anschließende Desulfonierung umfassenden Verfahren behandelt wurde.

9. Immunassay-Kit nach Anspruch 8, wobei das HCV-NS3-Protein zusätzlich mit einem zwitterionischen Detergenz behandelt wird.

10. Immunassay-Kit nach Anspruch 9, wobei das HCV-NS3-Protein mit Lauryldimethylbetain als zwitterionischem Detergenz behandelt wird.

11. Verfahren zur Herstellung eines Immunassay-Kits nach einem der Ansprüche 1 bis 10, wobei das Reduktionsmittel in wenigstens einem der unten aufgeführten Schritte vorhanden ist:

    (i) Beschichten der Festphase mit dem Antigen; und
    (ii) nach (i) Blockieren der Festphase; und
    (iii) nach (ii) Fixieren der auf der Festphase aufgebrachten Proteine; und
    (iv) nach (iii) Vorbehandeln der Festphase.

12. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritt (i) zugegeben wird.

13. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritt (ii) zugegeben wird.

14. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritten (i) und (ii) zugegeben wird.

15. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritt (iii) zugegeben wird.

16. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritten (i) und (iii) zugegeben wird.

17. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritt (iv) zugegeben wird.

18. Verfahren nach Anspruch 11, wobei das Reduktionsmittel in Schritten (i) und (iv) zugegeben wird.

19. Verfahren nach einem der Ansprüche 11 bis 18, wobei es sich bei dem Reduktionsmittel um DTT, DTE oder TCEP handelt.

20. Verfahren nach einem der Ansprüche 11 bis 19, wobei das Reduktionsmittel in einem Konzentrationsbereich von 0,1 mM bis 1 M, besonders von 0,5 mM bis 500 mM, ganz besonders von 1 mM bis 250 mM verwendet wird, wobei bei einigen Anwendungen Bereiche von 0,5 mM bis 50 mM, 1 bis 30 mM, 2 bis 20 mM oder 5 bis 15 mM oder etwa 10 mM erforderlich sein können.

21. Immunassay-Kit nach einem der Ansprüche 1 bis 3, bei dem es sich um einen ELISA-Kit, einen QUICK-Test-Kit oder einen Line-Immunassay-Kit handelt.

22. Verfahren nach Anspruch 11, wobei es sich bei dem hergestellten Immunassay-Kit um einen ELISA-Kit, einen QUICK-Test-Kit oder einen Line-Immunassay-Kit handelt.

23. Verwendung des Immunassay-Kits nach einem der Ansprüche 1 bis 3 zum Nachweis von Antikörpern gegen das HCV-NS3-Protein.

24. Verwendung des Immunassay-Kits nach Anspruch 23 zum Nachweis der Antikörper in einer biologischen Probe.

25. Verwendung des Immunassay-Kits nach einem der Ansprüche 1 bis 3 zum Nachweis der frühen HCV-NS3-Serokonversion.

**Revendications**

1. Trousse de dosage immunologique en phase solide comprenant, sur ladite phase solide, une protéine NS3 du HCV, ou un de ses analogues, en présence d'un agent réducteur, dans laquelle ladite protéine NS3 du HCV ou son analogue comprend une séquence d'acides aminés définissant au moins un épitope de la NS3 protéase ou hélicase du HCV.

2. Trousse de dosage immunologique comprenant une phase solide portant, comme antigène, une protéine NS3 du HCV, ou un de ses analogues, qui a été réduite par un agent réducteur, dans laquelle ladite protéine NS3 du HCV ou son analogue comprend une séquence d'acides aminés définissant au moins un épitope de la NS3 protéase ou hélicase du HCV.

3. Trousse de dosage immunologique comprenant une phase solide portant, comme antigène, une protéine NS3 du HCV, ou un de ses analogues, dans laquelle ladite protéine NS3 du HCV ou son analogue comprend une séquence d'acides aminés définissant au moins un épitope de la NS3 protéase ou hélicase du HCV, et dans laquelle ladite trousse a été produite par addition d'un agent réducteur dans au moins une des étapes ci-dessous :

(i) revêtement de ladite phase solide avec ledit antigène ;
(ii) blocage de ladite phase solide ;
(iii)fixation des protéines appliquées sur ladite phase solide ;
(iv) prétraitement de ladite phase solide.

4. Trousse de dosage immunologique selon l'une quelconque des revendications 1 à 3 dans laquelle ladite protéine NS3 du HCV est une séquence d'acides aminés de NS3 du HCV choisie dans le groupe constitué par SEQ ID n°: 3-18.

5. Trousse de dosage immunologique selon l'une quelconque des revendications 1 à 4 dans laquelle ladite protéine NS3 du HCV est contenue dans une protéine de fusion.

6. Trousse de dosage immunologique selon la revendication 5 dans laquelle ladite protéine de fusion est choisie dans le groupe des séquences d'acides aminés constituées de SEQ ID n°: 20, SEQ ID n°: 22, SEQ ID n°: 24, SEQ ID n°: 26, SEQ ID n°: 28, SEQ ID n°: 30 et SEQ ID n°: 32.

7. Trousse de dosage immunologique selon l'une quelconque des revendications 1 à 6 dans laquelle ladite protéine NS3 du HCV est une protéine NS3 hélicase du HCV ou une partie de celle-ci, contenant soit S1200, A1218, A1384, P1407, V1412, P1424 ou F1444, soit une combinaison d'un desdits acides aminés avec les acides aminés suivants, quels qu'ils soient, choisis dans le groupe constitué par L1201, S1222, 11274, S1289, T1321, A1323, T1369, L1382, V1408, A1409, F1410, et dans laquelle ladite protéine NS3 hélicase du HCV ou une partie de celle-ci comprend une séquence d'acides aminés définissant au moins un épitope de la NS3 protéase ou hélicase du HCV.

8. Trousse de dosage immunologique selon l'une quelconque des revendications 1 à 7 dans laquelle ladite protéine NS3 du HCV est une protéine NS3 du HCV traitée par un procédé comprenant les étapes de sulfonation, puis de désulfonation.

9. Trousse de dosage immunologique selon la revendication 8 dans laquelle ladite protéine NS3 du HCV est en plus traitée avec un détergent zwitterionique.

10. Trousse de dosage immunologique selon la revendication 9 dans laquelle ladite protéine NS3 du HCV est traitée avec de la lauryldiméthylbétaïne en tant que détergent zwitterionique.

11. Procédé de production d'une trousse de dosage immunologique selon l'une quelconque des revendications 1 à 10 dans lequel ledit agent réducteur est présent dans au moins une des étapes suivantes :

(i) revêtement de ladite phase solide avec ledit antigène ;
(ii) après (i), blocage de ladite phase solide ;
(iii)après (ii),fixation des protéines appliquées sur ladite phase solide ; et
(iv) après (iii), prétraitement de ladite phase solide.

12. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans l'étape (i).

13. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans l'étape (ii) .

14. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans les étapes (i) et (ii).

15. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans l'étape (iii).

16. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans les étapes (i) et (iii).

17. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans l'étape (iv).

18. Procédé selon la revendication 11 dans lequel ledit agent réducteur est ajouté dans les étapes (i) et (iv).

19. Procédé selon l'une quelconque des revendications 11 à 18 dans lequel ledit agent réducteur est le DTT, le DTE ou la TCEP.

20. Procédé selon l'une quelconque des revendications 11 à 19 dans lequel ledit agent réducteur est utilisé à une gamme de concentrations de 0,1 mM à 1 M, plus particulièrement de 0,5 mM à 500 mM, encore mieux de 1 mM à 250 mM, certaines applications pouvant nécessiter des gammes de 0,5 à 50 mM, de 1 à 30 mM, de 2 à 20 mM ou de 5 à 15 mM, ou environ 10 mM.

21. Trousse de dosage immunologique selon l'une quelconque des revendications 1 à 3 qui est une trousse ELISA, une trousse d'essai QUICK ou une trousse Line Immunoassay.

22. Procédé selon la revendication 11 dans lequel ladite trousse de dosage immunologique produite est une trousse ELISA, une trousse d'essai QUICK ou une trousse Line Immunoassay.

23. Utilisation de la trousse de dosage immunologique selon l'une quelconque des revendications 1 à 3 pour détecter les anticorps d'une protéine NS3 du HCV.

24. Utilisation de la trousse de dosage immunologique selon la revendication 23 pour détecter lesdits anticorps dans un prélèvement biologique.

25. Utilisation de la trousse de dosage immunologique selon l'une quelconque des revendications 1 à 3 pour détecter une séroconversion précoce de NS3 du HCV.

Figure 1 - 1

```
NS3A5         MGVAKAVDFIPVENLETTMRSPVFTDNSSPPAVPQSFQVAHLHAPTGSGKSTKVPAAYAA
NS3A26        ------------------------------------------------------T---V---
NS3B7         ----------S-----------------------------------------------G
NS3B9         ----------S-----------------------------------------------
NS3B12        ----------S-----------------------------------------------
NS3B14        ----------S-----------------------------------------------
NS3C1         ----------SM--------------------T-----------------------
NS3C3         ----------SM--------------------T----------S-----------
NS3C4         ----------SM--------------------T-----------------------
NS3C12        ----------SM--------------------T-----------------------
NS3C16        ----------SM--------------------T-----------------------
NS3D17        ------------------------------------------------------------
NS3D18        ------------------------------------------D-----------
NS3D19        ------------------------------------------------------------
NS3HCCL19A    ---------V---SM--------------------T-----------------------
NS3HCCL19B    ---------V---SM--------------------T-----------------------
```

Figure 1 - 2

```
NS3A5          QGFKVLVLNPSVAATLGFGAYMSRAHGIDPNIRTGVRTITTGSPITYSTYGKFLADGGCS
NS3A26         ------------------------------------------------------------
NS3B7          --Y----------L-----KV--------------------------------------
NS3B9          --Y----------------K--------------------------------------
NS3B12         --Y----------------K--------------------------------------
NS3B14         --Y-M----------K-Y----------T----------R-
NS3C1          --Y----------K------G----------A----------
NS3C3          --Y----------K------G----------A----------
NS3C4          --Y----------K------G----------A----------
NS3C12         --Y----------K------G----------A----------
NS3C16         --Y----------K------G----------A----------
NS3D17         --Y----------K---V--------------------------
NS3D18         --Y----------K---V----------N----
NS3D19         --Y----------K---V--------------------------
NS3HCCL19A     --Y----------K---V----------A----------
NS3HCCL19B     --Y----------K---V----------A----------
```

Figure 1 - 3

```
NS3A5          GGAYDIMICDECHSTDATSILGIGTVLDQAETAGARLVVLATAAPPGSVTVPHPNIEEVA
NS3A26         ------I----------------------------------T----------------
NS3B7          ------I----------------------------------T----------------
NS3B9          ------I----------------------------------T----------------
NS3B12         ------I-------------D--------------------T----------------
NS3B14         ------I----------------------------------T----------------
NS3C1          ------I---------S-T----------------------T----------------
NS3C3          ------I---------S-T----------------------T---------------
NS3C4          ------I---------S-T----------------------T----------------
NS3C12         ------I---------S-T----------------------T----------------
NS3C16         ------I---------S-T----------------------T----------------
NS3D17         ------I---------V----------------------T----------------
NS3D18         ------I---------V----------------------T----------------
NS3D19         ------I---------V----------------------T----------------
NS3HCCL19A     ------I-------I-S----------------------T----------------
NS3HCCL19B     ------I-------I-S----------------------T----------------
```

Figure 1 - 4

```
NS3A5         LSTTGEIPFYGKAIPLEAIKGGRHLIFCHSKKKCDELAAKLTALGVNAVAYYRGLDVSVI
NS3A26        ------------------------R-----------------------------------
NS3B7         ---------C-----------------------------N-V-----P------------
NS3B9         ----------------------------------------PV---------------P--
NS3B12        ----------------------------------------V------------------
NS3B14        -------------------------V---------------V------------------
NS3C1         --N--------------T-----------------------SS--L--------------
NS3C3         --N--------------T-----------------------SS--I--------------
NS3C4         --N--------------T-----------------------SS--L--------------
NS3C12        --N--------------T-----------------------SS--L--------------
NS3C16        --N--------------T-----------------------SS--L--------------
NS3D17        ------------------------------------K-----V---I--------------
NS3D18        ----------------------------------------V---I----------------
NS3D19        ----------------------------------------QV---I--------------
NS3HCCL19A    --SI-----------I-V-----------------------SGV-I--------------
NS3HCCL19B    --S------------I-V-----------------------SGF-I--------------
```

Figure 1 - 5

| | | |
|---|---|---|
| NS3A5 | PTSGDVVVVATDALMTGYTGDFDSVIDCNTCVTQTVDFS | (SEQ ID NO 3) |
| NS3A26 | ---------- | (SEQ ID NO 4) |
| NS3B7 | ---------- | (SEQ ID NO 5) |
| NS3B9 | ----------F---------- | (SEQ ID NO 6) |
| NS3B12 | ----------F---------- | (SEQ ID NO 7) |
| NS3B14 | ----------F----------R | (SEQ ID NO 8) |
| NS3C1 | ----------F----------A------- | (SEQ ID NO 9) |
| NS3C3 | ----------F----------A------- | (SEQ ID NO 10) |
| NS3C4 | ----------F---------- | (SEQ ID NO 11) |
| NS3C12 | ----------F---------- | (SEQ ID NO 12) |
| NS3C16 | ----------F---------- | (SEQ ID NO 13) |
| NS3D17 | ----------I- | (SEQ ID NO 14) |
| NS3D18 | ---------- | (SEQ ID NO 15) |
| NS3D19 | ---------- | (SEQ ID NO 16) |
| NS3HCCL19A | ----------F---------- | (SEQ ID NO 17) |
| NS3HCCL19B | ----------F---------- | (SEQ ID NO 18) |

Figure 2-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GGGGTTGCGAAGGCGGTGGACTTTGTACCCGTAGAGTCTATGGAAACCACCATGCGGTCC
CCGGTCTTTACGGATAACTCATCTCCTCCGGCCGTACCGCAGACATTCCAAGTGGCCCAT
CTACACGCCCCCACTGGTAGTGGCAAGAGCACTAAGGTGCCGGCTGCATATGCAGCCCAA
GGGTACAAGGTACTTGTCCTGAACCCATCCGTTGCCGCCACCTTAGGATTCGGGGCGTAT
ATGTCTAAAGCACATGGTGTCGACCCTAACATTAGAACTGGGGTAAGGACCATCACCACG
GGCGCCCCCATTACGTACTCCACCTACGGCAAGTTTCTTGCCGACGGTGGTTGCTCTGGG
GGCGCTTACGACATCATAATATGTGATGAGTGCCACTCGATTGACTCAACCTCCATCTTG
GGCATCGGCACCGTCCTGGATCAGGCGGAGACGGCTGGAGCGCGGCTTGTCGTGCTCGCC
ACTGCTACACCTCCGGGGTCGGTCACCGTGCCACATCCCAACATCGAGGAGGTGGCTCTG
TCCAGCACTGGAGAGATCCCCTTTTATGGCAAAGCCATCCCCATCGAGGTCATCAAAGGG
GGGAGGCACCTCATTTTCTGCCATTCCAAGAAGAAATGTGACGAGCTCGCCGCAAAGCTA
TCGGGCTTCGGAATCAACGCTGTAGCGTATTACCGAGGCCTTGATGTGTCCGTCATACCG
ACTAGCGGAGACGTCGTTGTTGTGGCAACAGACGCTCTAATGACGGGCTTTACCGGCGAC
TTTGACTCAGTGATCGACTGTAACACATGCGTCACCCAGACAGTCGACTTCAGCTAA

(SEQ ID NO 19)

Figure 2-2

**MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPM**GVAKAVDFVPVESMETTMRSPVFTD
NSSPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHGVDPN
IRTGVRTITTGAPITYSTYGKFLADGGCSGGAYDIIICDECHSIDSTSILGIGTVLDQAETAGAR
LVVLATATPPGSVTVPHPNIEEVALSSTGEIPFYGKAIPIEVIKGGRHLIFCHSKKKCDELAAKL
SGFGINAVAYYRGLDVSVIPTSGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDFS

(SEQ ID NO 20)

Figure 3-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAACCACCA
AGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATGGGGGTTGCGA
AGGCGGTGGACTTTATCCCCGTGGAGAGCCTAGAAACAACCATGAGGTCCCCGGTGTTCACAGAC
AACTCCTCCCCGCCAGCAGTGCCCCAGAGCTTCCAGGTGGCCCACCTGCATGCTCCCACCGGCAG
CGGTAAGAGCACCAAGGTCCCGGCCGCATATGCGGCTCAGGGCTACAAAGTGCTGGTGCTCAACC
CCTCCGTTGCTGCAACATTGGGCTTTGGTGCTTACATGTCCAAGGCCCATGGGATTGATCCTAAC
ATCAGGACTGGGGTAAGGACAATTACTACTGGCAGCCCCATCACGTACTCCACCTACGGCAAGTT
CCTTGCCGACGGCGGGTGCTCGGGGGGTGCTTATGACATAATAATTTGTGACGAGTGCCACTCCA
CAGATGCAACATCTATTTTGGGCATCGGCACTGTCCTTGACCAAGCAGAGACTGCGGGGGCGAGA
CTGGTTGTGCTTGCCACCGCTACCCCTCCGGGCTCCGTCACTGTGCCCCATCCTAATATCGAGGA
GGTTGCTCTGTCCACCACCGGAGAGATCCCCTTTTACGGCAAGGCTATCCCCCTTGAGGCAATCA
AAGGGGGGAGACATCTCATCTTCTGCCACTCAAAGAAGAAGTGCGACGAACTCGCCGCCAAACCG
GTCGCGTTGGGTGTCAATGCCGTGGCTTACTACCGCGGCCTTGACGTGCCCGTCATCCCGACCAG
TGGCGATGTTGTCGTCGTGGCAACTGATGCTCTCATGACCGGTTTTACCGGTGACTTCGACTCGG
TGATAGACTGTAATACGTGTGTCACCCAGACAGTCGACTTCAGCTAA

(SEQ ID NO 21)

Figure 3-2

**MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHVDPGPM**GVAKAVDFIPVESLETTMRSPVFTD
NSSPPAVPQSFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHGIDPN
IRTGVRTITTGSPITYSTYGKFLADGGCSGGAYDIIICDECHSTDATSILGIGTVLDQAETAGAR
LVVLATATPPGSVTVPHPNIEEVALSTTGEIPFYGKAIPLEAIKGGRHLIFCHSKKKCDELAAKP
VALGVNAVAYYRGLDVPVIPTSGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDFS

(SEQ ID NO 22)

Figure 4-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GCCGCGGGATTGGGCCCCCCCATAGGTGTAGCAAAAGCCCTACAGTTCATACCAGTGGAA
ACCCTTAGTACGCAGGCTAGGTCTCCATCTTTCTCTGACAATTCAACTCCTCCTGCTGTC
CCACAGAGCTATCAAGTAGGGTATCTTCATGCCCCGACCGGCAGCGGTAAGAGCACAAAG
GTCCCGGCCGCTTATGTAGCACAAGGATATAATGTTCTCGTGCTGAATCCATCGGTGGCG
GCCACACTAGGCTTCGGCTCTTTCATGTCGCGTGCCTATGGGATCGACCCCAACATCCGC
ACTGGGAACCGCACCGTCACAACTGGTGCTAAACTGACCTATTCCACCTACGGTAAGTTT
CTCGCGGACGGGGGTTGCTCCGGGGGGGCATATGATGTAATTATCTGTGATGAATGTCAT
GCCCAAGACGCCACTAGCATATTGGGCATAGGCACGGTCTTAGATCAGGCCGAGACGGCT
GGGGTGAGGCTCACGGTTTTAGCGACAGCAACTCCCCCAGGCAGCATCACTGTGCCACAT
TCTAACATCGAGGAAGTGGCCCTGGGCTCTGAAGGTGAGATCCCTTTCTACGGTAAGGCT
ATACCGATAGCCCTGCTCAAGGGGGGGAGACACCTCGTCTTTTGCCATTCCAAGAAAAAA
TGTGATGAGCTAGCATCCAAACTCAGAGGTATGGGGCTCAACGCTGTGGCGTACTATAGG
GGTCTCGATGTTTCCGTCATACCAACAACAGGAGACGTCGTGGTCTGCGCTACTGACGCC
CTCATGACTGGATTCACTGGAGACTTCGATTCTGTCATAGACTGCAACGTGGCTGTTGAA
CAGTACGTTGACTTCAGCTAA

(SEQ ID NO 23)

Figure 4-2

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMAAGLGPPIGVAKALQFIPVE
TLSTQARSPSFSDNSTPPAVPQSYQVGYLHAPTGSGKSTKVPAAYVAQGYNVLVLNPSVA
ATLGFGSFMSRAYGIDPNIRTGNRTVTTGAKLTYSTYGKFLADGGCSGGAYDVIICDECH
AQDATSILGIGTVLDQAETAGVRLTVLATATPPGSITVPHSNIEEVALGSEGEIPFYGKA
IPIALLKGGRHLVFCHSKKKCDELASKLRGMGLNAVAYYRGLDVSVIPTTGDVVVCATDA
LMTGFTGDFDSVIDCNVAVEQYVDFS

(SEQ ID NO 24)

Figure 5-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GCCGCGGGATTGGGCCCCACCATAGGTGTAGCAAAAGCCCTACAGTTCATACCAGTGGAA
ACCCTTAGCACACAGGCTAGGTCTCCATCTTTCTCTGACAATTCAACTCCTCCTGCTGTT
CCACAGAGCTATCAAGTAGGGTACCTTCATGCCCCGACCGGCAGCGGTAAGAGCACAAAG
GTCCCGGCCGCTTATGTAGCACAAGGATATACTGTTCTCGTGCTGAATCCATCGGTGGCG
GCCACACTAGGCTTCGGCTCTTTCATGTCGCGTGCCTATGGGATCGACCCCAACATCCGC
ACTGGGAACCGCACCGTTACAACTGGTGCTAAACTGACCTATTCCACCTACGGTAAGTTT
CTTGCGGATGGGGGTTGCTCCGGGGGGGGCATATGATGTGATTATCTGTGATGAGTGTCAT
GCCCAAGACGCTACTAGCATATTGGGTATAGGCACGGTCTTAGATCAGGCCGAGACGGCT
GGGGTGAGGCTGACGGTTTTAGCGACAGCGACCCCCCCAGGCAGCATCACTGTGCCACAT
TCTAACATCGAAGAAGTGGCCCTGGGCTCTGAGGGTGAGATCCCCTTCTACGGCAAGGCT
ATACCGATATCCCTGCTCAAGGGGGGGAGGCACCTTATCTTTTGCCATTCCAAAAAAAAG
TGTGATAAGATAGCGTCCAAACTCAGAGGCATGGGGCTCAACGCTGTAGCGTACTATAGA
GGTCTCGATGTGTCCGTCATACCAACAACAGGAGACGTCGTAGTTTGCGCTACTGACGCC
CTCATGACTGGATACACCGGGGACTTCGATTCTGTCATAGACTGCAACGTGGCTGTTGAA
CAGTACGTTGACTTCAGCTAA

(SEQ ID NO 25)

Figure 5-2

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMAAGLGPTIGVAKALQFIPVE
TLSTQARSPSFSDNSTPPAVPQSYQVGYLHAPTGSGKSTKVPAAYVAQGYTVLVLNPSVA
ATLGFGSFMSRAYGIDPNIRTGNRTVTTGAKLTYSTYGKFLADGGCSGGAYDVIICDECH
AQDATSILGIGTVLDQAETAGVRLTVLATATPPGSITVPHSNIEEVALGSEGEIPFYGKA
IPISLLKGGRHLIFCHSKKKCDKIASKLRGMGLNAVAYYRGLDVSVIPTTGDVVVCATDA
LMTGYTGDFDSVIDCNVAVEQYVDFS

(SEQ ID NO 26)

Figure 6-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GGCGTGGCCAAGTCCATAGACTTCATCCCCGTTGAGACACTCGACATCGTTACGCGGTCC
CCCACCTTTAGTGACAACAGCACGCCACCGGCTGTGCCCCAGACCTATCAGGTCGGGTAC
TTGCATGCCCCAACCGGCAGCGGAAAGAGCACCAAAGTCCCCGTCGCATACGCCGCCCAG
GGGTATAAAGTGTTAGTGCTCAATCCCTCGGTGGCTGCTACCCTGGGGTTTGGAGCGTAC
CTGTCCAAGGCACACGGCATCAATCCCAACATTAGGACTGGAGTCAGGACTGTGACGACT
GGCGAAGCCATCACGTACTCCACGTATGGCAAATTCCTCGCCGATGGGGGCTGCGCAGGT
GGCGCCTATGACATCATCATATGCGATGAATGCCACGCCGTGGATGCCACTACCATTCTC
GGCATCGGAACAGTCCTTGACCAAGCAGAGACAGCCGGGGTCAGGCTAACTGTGCTGGCT
ACGGCCACGCCCCCCGGGTCAGTGACAACCCCCCATCCCAACATAGAGGAGGTAGCCCTC
GGGCAGGAGGGTGAGACCCCCTTCTATGGGAGGGCGATCCCCCTGTCTTACATCAAGGGA
GGGAGACACTTGATCTTCTGCCACTCAAAGAAAAAGTGTGACGAGCTCGCGGCGGCCCTC
CGGGGCATGGGCCTGAACGCTGTGGCGTACTACAGAGGGCTCGACGTCTCCGTAATACCA
GCTCAGGGAGATGTAGTGGTCGTCGCCACCGACGCCCTCATGACGGGGTTCACTGGAGAC
TTTGACTCCGTGATCGACTGCAATGTAGCGGTCACTCAAGTTGTAGACTTCAGCTAA

(SEQ ID NO 27)

Figure 6-2

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHVDPGPMGVAKSIDFIPVETLDIVTRS
PTFSDNSTPPAVPQTYQVGYLHAPTGSGKSTKVPVAYAAQGYKVLVLNPSVAATLGFGAY
LSKAHGINPNIRTGVRTVTTGEAITYSTYGKFLADGGCAGGAYDIIICDECHAVDATTIL
GIGTVLDQAETAGVRLTVLATATPPGSVTTPHPNIEEVALGQEGETPFYGRAIPLSYIKG
GRHLIFCHSKKKCDELAAALRGMGLNAVAYYRGLDVSVIPAQGDVVVVATDALMTGFTGD
FDSVIDCNVAVTQVVDFS

(SEQ ID NO 28)

Figure 7-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GGCGTAGCCAAATCCATTGACTTCATCCCTGTTGAATCTCTCGATATCGCCTCACGGTCA
CCCAGTTTCTCTGACAACAGCACGCCACCAGCTGTGCCTCAGTCCTACCAGGTGGGCTAT
TTGCACGCGCCAACGGGCAGCGGGAAGAGCACCAAGGTCCCTGTCGCATATGCTAGTCAG
GGGTATAAAGTACTCGTGCTAAATCCCTCTGTCGCGGCCACGCTCGGCTTCGGGGCCTAC
ATGTCCAAAGCCCACGGGATCAACCCCAACATCAGAACCGGGGTACGGACTGTGACCACC
GGGGACCCCATCACCTACTCCACTTATGGCAAGTTTCTCGCAGATGGGGGCTGCTCAGCC
GGCGCCTATGATGTCATCATATGCGATGAATGCCACTCAGTGGACGCTACTACCATCCTT
GGCATTGGAACAGTCCTCGACCAGGCCGAGACCGCGGGTGCTAGGTTAGTGGTTTTAGCC
ACAGCCACGCCTCCTGGTACAGTGACAACTCCTCATAGCAACATAGAGGAGGTGGCTCTT
GGTCATGAAGGCGAGATCCCTTTCTACGGCAAGGCTATTCCCCTAGCTTTCATCAAGGGG
GGCAGACACCTAATCTTTTGCCATTCAAAGAAGAAGTGCGATGAGCTCGCGGCAGCCCTT
CGGGGCATGGGTGTCAACGCCGTTGCTTACTATAGGGGTCTCGACGTCTCTGTTATACCA
ACTCAAGGAGACGTGGTGGTCGTTGCCACCGATGCCCTAATGACTGGATACACCGGTGAC
TTTGACTCTGTTATTGACTGCAACGTTGCGGTCTCTCAAATTGTAGACTTCAGCTAA

(SEQ ID NO 29)

Figure 7-2

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMGVAKSIDFIPVESLDIASRS
PSFSDNSTPPAVPQSYQVGYLHAPTGSGKSTKVPVAYASQGYKVLVLNPSVAATLGFGAY
MSKAHGINPNIRTGVRTVTTGDPITYSTYGKFLADGGCSAGAYDVIICDECHSVDATTIL
GIGTVLDQAETAGARLVVLATATPPGTVTTPHSNIEEVALGHEGEIPFYGKAIPLAFIKG
GRHLIFCHSKKKCDELAAALRGMGVNAVAYYRGLDVSVIPTQGDVVVVATDALMTGYTGD
FDSVIDCNVAVSQIVDFS

(SEQ ID NO 30)

Figure 8-1

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAAC
CACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCACGTGGATCCCGGGCCCATG
GGCGTAGCCAAATCCATTGACTTCATCCCCGTTGAGTCTCTCGACATCGTGACTAGGTCT
CCAAGCTTCACTGACAACAGTACACCTCCAGCCGTGCCTCAGACCTACCAAGTGGGGTAT
CTCCACGCGCCCACTGGTAGCGGGAAGAGTACCAAGGTCCCTGCAGCGTACGCCGCTCAG
GGGTACAAGGTGCTGGTACTGAACCCCTCCGTGGCTGCCACTTTGGGATTTGGGGCCTAC
ATGTCAAAAGCGCACGGAGTCAATCCCAATATCAGGACCGGGGTTCGCACGGTGAACACT
GGGGATCCCATCACCTACTCCACGTATGGCAAATTCCTCGCAGATGGAGGCTGCTCTGGA
GGCGCCTATGGCATCATAATATGCGACGAATGCCATTCGACGGACTCCACGACCATCCTC
GGCATCGGGACCGTTCTCGACCAAGCTGAGACAGCTGGAGTTAGGTTGGTGGTCTTGGCC
ACGGCGACCCCACCCGGATCTGTAACAACCCCACACCCCAACATAGAGGAGGTGGCCCTC
GGCCACGAGGGCGAAATCCCCTTCTATGGGAAGGCCATCCCTCTCTCAACCATCAAGGGA
GGACGACATCTAATCTTCTGTCATTCAAAGAAAAAGTGCGACGAGCTCGCGGTGGCCCTC
CGAGCGATGGGCCTTAACGCGGTGGCATACTACAGAGGGCTTGACGTCTCCGTGATACCA
ACACAAGGAGACGTGGTGGTCGTCGCCACCGACGCCCTCATGACAGGATATACTGGAGAC
TTCGACTCTGTGATCGACTGCAACATGGCGGTCTCTCAAATTGTAGACTTCAGCTAA

(SEQ ID NO 31)

**Figure 8-2**

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMGVAKSIDFIPVESLDIVTRS
PSFTDNSTPPAVPQTYQVGYLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAY
MSKAHGVNPNIRTGVRTVNTGDPITYSTYGKFLADGGCSGGAYGIIICDECHSTDSTTIL
GIGTVLDQAETAGVRLVVLATATPPGSVTTPHPNIEEVALGHEGEIPFYGKAIPLSTIKG
GRHLIFCHSKKKCDELAVALRAMGLNAVAYYRGLDVSVIPTQGDVVVVATDALMTGYTGD
FDSVIDCNMAVSQIVDFS

(SEQ ID NO 32)