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(54) **Title:** WOUND PROGNOSIS

(57) **Abstract:** A method of wound prognosis comprising the steps of: determining whether a weighted average level of human neutrophil elastase (hNE) and one or more matrix metalloproteinase (MMP) in a sample of wound fluid from a wound exceeds a predetermined threshold value for said weighted average; and assigning said wound to a non-healing category if said weighted average exceeds said predetermined threshold value for said weighted average. Also provided are devices for performing the method.



WOUND PROGNOSIS

The present invention relates to methods and devices for determining the healing status of wounds, in particular of chronic wounds. Also provided are kits comprising the diagnostic
5 devices, and methods of wound diagnosis and treatment using the diagnostic devices and methods.

The word 'chronic' is a generic term used to describe a condition that is 'of long duration' or that 'recurs frequently'. It is for this reason it is used to describe wounds that are difficult to
10 heal and usually persist for a long period of time. Thus, while all wounds have the potential to become chronic, traditionally chronic wounds are identified by their underlying aetiology such as venous insufficiency, arterial perfusion, diabetes, or unrelieved pressure as well as systemic factors such as nutritional status, immunosuppression, and infection all of which may contribute to poor wound healing. In general it is accepted that acute wounds follows an
15 orderly sequence of events and typically heal within 2-4 weeks, whereas a chronic wound is thought to be stuck in the inflammatory phase of healing and can therefore persist for months or even years. Chronic wounds have been shown to contain elevated levels of inflammatory cytokines, free radicals and proteases which create a hostile wound environment, detrimental to healing.

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Predicting the healing status of a chronic wound is difficult, and is often complicated by the number of risk factors associated with these wounds. The most commonly used objective measure is a reduction of the ulcer surface area. This was ratified by The European Wound Management Association whom produced a review of the different outcome measures used in
25 controlled and comparative wound healing studies. In this study, they reported that the most frequently used outcome was wound surface area reduction and that this was a valid end point for such studies. It has been shown that a reduction in wound area after two to four weeks is a good predictor of the wound's ability to heal by week twelve. It is harder to determine from the literature the exact percentage reduction which should be used to predict healing status
30 however it is generally accepted that for venous leg ulcers a 20-40% reduction in wound area within 2-4 weeks is predictive of healing, whereas for diabetic foot ulcers a reduction of 50% within the first 4 weeks is predictive of healing.

It would be desirable to be able to determine whether a chronic wound is healing, or not, by
35 means of a simple test that does not depend on the measurement of wound surface area over a

period of two to four weeks. Such a test would enable the appropriate therapy to be selected as early as possible during wound treatment. If the wound is non-healing, then special dressings or other therapies can be used as early as possible and/or an ineffective therapy can be changed. If the wound is found to be healing without special intervention, then the additional
5 expense of special therapies can be avoided.

BACKGROUND ART

Many documents describe the measurement of various different analytes in chronic and acute wound fluid. The following documents describe the measurement of endogenous protease
10 enzymes in wound fluid.

C.N. Rao *et al.* in the Journal of Investigative Dermatology, vol. 105(4), pages 572-578 (1995) describe the results of analysing chronic and acute wound fluids for elastase, alpha-1-antitrypsin (AAT) and fibronectin. It was found that the elastase level was 10 to 40 times
15 higher in the chronic wound fluid.

US-A-2003/0119073 describes sensors for the assay of catabolic protease enzymes in wound fluid. The analyte enzymes include human neutrophil elastase (hNE). It is suggested therein that the invention can be used in a method of treating chronic wounds by detecting the
20 presence of catabolic protease enzymes, and then treating the wound with inhibitors that are specific for the detected enzymes.

WO 2006/030232 describes a diagnostic test apparatus for determining a ratio of: (a) at least one endogenous protease enzyme inhibitor, to (b) at least one endogenous protease enzyme, in
25 a sample of a wound fluid. The apparatus is used for identifying wounds that are likely to respond well to treatment with oxidized regenerated cellulose (ORC) containing dressings.

John F. Tarlton *et al.* in Wound Repair and Regeneration, Vol. 7(5), pages 347-355 (1999) describe an academic study into the prognostic value of markers of collagen remodelling in
30 venous ulcers. The following markers were studied: proMMP-2, proMMP-9, neutrophil elastase (hNE), MMP-2 and MMP-9. The data showed that expression of MMPs-2 and 9 and hNE were higher in venous ulcer exudates than in acute wound fluids. Also studied was a comparison of the above markers in healing versus non-healing ulcers. A further study was performed in which wound fluids were compared from healing and non-healing

regions of the same ulcer in order to eliminate patient-to-patient variation. The data showed only very weak correlation, if any, of MMP-2 and MMP-9 to wound healing status in the multiple-patient study. The correlation for neutrophil elastase was also quite weak. However, the comparison experiments done on wound fluids from different regions of the same wound did show a statistically significant correlation of neutrophil elastase to wound healing status, and a weakly statistically significant correlation of MMP-9 to wound healing status.

Alison G. Patrick *et al.* in *Macromolecular Bioscience*, Vol. 10, pages 1184-1193 (2010) describe hydrogel particles having surface bonded moieties for the detection of elastase, MMP-1 and/or MMP-12. The moieties consist of an enzyme-specific peptide substrate linking two FRET chromophores, whereby cleavage of the peptide results in a fluorescence signal. The peptides are selective for elastase, or for MMP-1 and MMP-12. It is mentioned that MMPs are associated with the progression of chronic wounds.

Ralf Smeets *et al.* in *International Wound Journal*, Vol. 5(2), pages 195-203 (2008) describe the effect of collagen/ORC sponge dressing treatment on "gelatinase activity", and also on levels of MMP-2, and elastase in chronic wounds. The results show that collagen/ORC does not significantly effect the MMP-2 level, but that it significantly reduces the gelatinase and elastase activities.

Cornelia Wiegand *et al.* in *Archives of Dermatological Research*, Vol. 302, pages 419-428 (2010) describe measurement of various proteases in acute chronic wound fluids. The measured proteases were neutrophil elastase, MMP-2 and MMP-13. These were all found to be elevated in chronic wounds relative to acute wounds. Levels of these proteases were found to decrease in the course of wound healing.

Breda Cullen *et al.* in *Wound Repair and Regeneration*, Vol. 15 (6) page A148 (2007) describe a comparison of collagen/ORC/silver dressings with other collagen (+silver) dressings in the treatment of chronic wounds. The results demonstrate that while the collagen containing dressings could remove MMP-type proteases, not all inactivate elastase. However, the collagen/ORC/silver dressings significantly reduced the levels of both elastase and total MMP.

WO-A-03058237 describes sensors for detection of various protease enzymes in wound fluid. The analytes include MMP-1, MMP-8, MMP-9 and neutrophil elastase, either singly or simultaneously. The specification also suggests then treating the wound with inhibitors
5 that are specific for protease enzymes found in the wound. The sensors described in the specification are all immunological.

GB-A-2422664 refers to measurement of various analytes, including MMP-1, MMP-8, MMP-9 and neutrophil elastase in wound fluid. Peptide sequences are disclosed that are
10 cleavable by elastase, MMP-2 or MMP-9. Also disclosed are devices with multiple lateral flow paths for measuring multiple analytes.

WO-A-2008070865 describes methods for evaluating the status of the healing process of a wound comprising contacting wound fluid with a cleavable peptide. The method may be
15 used to detect at least one protease selected from the group of MMP-2, MMP-8, MMP-9 and elastase.

It will be apparent from the foregoing that levels of matrix metalloproteinases and human neutrophil elastase levels are thought to be elevated in chronic wound fluid relative to acute
20 wound fluid. Changes in the levels of these markers may be correlated to wound healing. However, the above references do not show how to use these markers to distinguish between healing and non-healing chronic wounds. For example, Tarlton *et al.* found little or no statistically significant correlation between these markers and healing/non-healing chronic wound type in inter-patient studies. Furthermore, protease levels in any sample of wound fluid
25 appear to be poorly correlated. Thus, a wound having high levels of hNE may not also have elevated levels of MMP. This may be due to interactions between proteases. For example hNE acts as a physiological activator of MMPs including MMP-9 which is synthesised in a latent, inactive form. Additionally, there is some biochemical redundancy between the proteases; although some proteases are more efficient than others there is a crossover and
30 synergy of substrates between these proteases, for example elastin can be degraded by both MMP-9 and Elastase, and both MMP-8 and MMP-9 are required to digest Collagen type I. Therefore it may be possible for a non-healing wound to be low in one protease but this could be compensated by an excess of another protease.

Thus, it is an object of the present invention to provide improved means to distinguish healing from non-healing chronic wounds. It is a further object to provide such a means that can be performed on a sample of wound fluid removed from the wound. It is a further object to provide a method that requires only a measurement made at a single point in time, in contrast to the measurement of wound area, which requires multiple measurements over an extended period. It is a further object of the invention to avoid unnecessary special therapy on other patients who present with healing wounds. Preferably, the present invention seeks to identify a sub-group of patients wherein the probability of their wound being non-healing is at least about 80%, suitably at least about 90%.

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SUMMARY OF THE INVENTION

The present inventors have found that non-healing wounds can be distinguished from healing wounds by measuring both human neutrophil elastase (hNE) activity and the activity of at least one matrix metalloproteinase (MMP) in a sample of the wound fluid, and comparing these measured values with one or more threshold values indicative of non-healing wounds. The measurement of both analyte types compensates for inter-patient variations in the individual analytes and thereby reduces false negative results. The selection of a sufficiently high threshold reduces false positive results. Overall, at least 80% or 90% of the wounds giving a positive result in this test are found to be non-healing.

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In a first aspect the present invention provides a method of wound prognosis comprising the steps of: determining whether a weighted average level of human neutrophil elastase (hNE) and one or more matrix metalloproteinase (MMP) in a sample of wound fluid from a wound exceeds a predetermined threshold value for said weighted average; and assigning said wound to a non-healing category if said weighted average exceeds said predetermined threshold value for said weighted average.

In a second aspect the present invention provides a device for simultaneously or sequentially measuring the levels of hNE and of at least one MMP in a sample of wound fluid, and for providing a detectable output if a weighted average level of said hNE and at least one MMP exceeds a predetermined threshold.

The present invention further provides a device for use in a method of wound prognosis comprising the steps of: determining a weighted average (weighted sum) of the levels of human neutrophil elastase (hNE) and at least one matrix metalloproteinase (MMP) in a sample

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of wound fluid from a wound; and assigning said wound to a non-healing category if said weighted average (weighted sum) exceeds a threshold value.

In a further aspect, the present invention provides a method of wound prognosis comprising the steps of: measuring the level of human neutrophil elastase (hNE) in a sample of wound fluid from a wound; measuring the level of at least one matrix metalloproteinase (MMP) in a sample of wound fluid from said wound; and assigning said wound to a non-healing category if either said level of hNE exceeds a first predetermined threshold or said level of MMP exceeds a second predetermined threshold. The measurement of hNE and one or more MMPs may be carried out simultaneously or sequentially in one or more diagnostic devices. In certain embodiments a single measurement is used to determine a sum or weighted average of hNE and one or more MMP levels.

In a further aspect the present invention provides a device for simultaneously or sequentially measuring the levels of hNE and of at least one MMP in a sample of wound fluid, and for providing a detectable output if either the level of the hNE exceeds a first predetermined threshold or the level of said at least one MMP exceeds a second predetermined threshold.

Suitably, the methods of the present invention may further comprise the step of determining said threshold value(s).

Suitably those methods according to certain aspects of the present invention comprising the step of determining whether a weighted average level of human neutrophil elastase (hNE) and one or more matrix metalloproteinase (MMP) in a sample of wound fluid from a wound exceeds a predetermined threshold value for said weighted average, may further comprise determining said threshold value by the steps of: determining from measurements of wound area versus time those wounds in a population of chronic wounds which are healing and which are non-healing; determining the weighted average level of hNE and at least one MMP in wound fluid samples taken from each of the healing and non-healing wounds; and selecting said threshold value such that the threshold identifies the largest number of non-healing wounds, provided that at least about 80% of the wounds falling above said threshold is non-healing.

Suitably, the population comprises at least about 30 healing wounds and at least about 30 non-healing wounds. Suitably, at least about 90% of the wounds falling above said threshold is non-healing.

- 5 Suitably those methods according to certain aspects of the present invention comprising the steps of measuring the level of human neutrophil elastase (hNE) in a sample of wound fluid from a wound; measuring the level of at least one matrix metalloproteinase (MMP) in a sample of wound fluid from said wound; and assigning said wound to a non-healing category if either said level of hNE exceeds a first predetermined threshold or said level of MMP
- 10 exceeds a second predetermined threshold, may further comprise determining said first and second thresholds by the steps of: determining from measurements of wound area versus time which wounds in a population of chronic wounds are healing and which are non-healing; determining the levels of hNE and MMP in wound fluid samples taken from each of the healing and non-healing wounds; and selecting said first and second thresholds such that the
- 15 thresholds identify the largest number of non-healing wounds provided that at least about 80% of the wounds falling above one or other of said thresholds is non-healing.

The term “level” may refer to the amount of the protease as determined by immunoassay, which may detect both active and inactive forms of the enzyme. More suitably, the term

20 “level” refers to the activity of the protease, for example as determined by the rate of cleavage of a substrate for the protease.

The at least elastase and at least one matrix metalloproteinase are suitably endogenous proteases present in the chronic wound fluid.

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In the above aspects or embodiments, the level of elastase may be indicated by total elastase-like activity, for example by measuring the rate of cleavage of a substrate for the hNE enzyme.

Suitably, the at least one matrix metalloproteinase includes MMPs selected from the group

30 consisting of MMP-1, MMP-8, MMP-9 and MMP-12. Typically, the matrix metalloproteinases include MMP-1 and MMP-9. The level of more than one matrix metalloproteinase may be measured. In these embodiments, the relevant level for comparison with the threshold is the sum of the levels of the measured MMPs. In certain embodiments, the at least one matrix metalloproteinase is at least two matrix metalloproteinases. In certain embodiments, the at

least one MMP is total MMP of all types in the sample. In certain embodiments, the level of at least one MMP is a weighted average (weighted sum) of the levels of more than one MMP.

The level of MMP may be determined by measuring the rate of cleavage of one or more
5 substrates for the MMP enzymes. Certain peptide substrates are specific for one or more MMPs. As explained further below, certain peptide substrates are cleaved by more than one MMP. In these embodiments, the rate of cleavage may depend on the MMP, whereby the rate of cleavage is determined by the respective activities of the MMPs in the sample weighted by their respective reactivities with the substrate. The resulting weighted average (weighting
10 sum) MMP activity is used as the measured level for comparison with a threshold value.

Likewise, in certain embodiments, a peptide substrate is cleaved both by hNE and by one or more MMPs. In these embodiments the rate of cleavage is determined by the respective activities of the hNE and the MMPs in the sample weighted by their respective reactivities with
15 the substrate. The resulting weighted average (weighted sum) protease activity is used as the measured level for comparison with a threshold value to determine whether the wound is healing or non-healing.

Accordingly, in a further aspect the present invention provides a method of wound prognosis
20 comprising the steps of: determining a weighted average (weighted sum) of the levels of human neutrophil elastase (hNE) and two or more matrix metalloproteinases (MMP); and assigning said wound to a non-healing category if said weighted average exceeds a threshold level.

25 The weighted average (weighted sum) is suitably weighted by the respective reactivities of the hNE and MMPs with one or more cleavable substrates for these protease enzymes.

In embodiments of the present invention according to any aspect, the proteases determined
include hNE, MMP-1 and MMP-9.

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In embodiments, the selective reagent, such as a cleavable exogenous peptide substrate, is immobilized in the device, for example by chemical or physical bonding to a solid substrate in said device, as described in more detail below. In embodiments, the selective reagent is conjugated to an indicator moiety or a binding moiety to form a selective reagent-moiety

conjugate that may or may not be bonded to a solid substrate in the measurement device of the invention.

Output signals may be of any suitable form as apparent to the skilled person. Suitable signals are disclosed herein. For example, output signals may be visual or auditory, and may be immediately recognisable (*e.g.* written text) or may require further interpretation by reference to a standard (*e.g.* a colour signal). In particular embodiments, the device is adapted to provide a visual output in the form of a colour line on a test strip, wherein the intensity of the colour line is indicative of the level of the protease analyte(s) in the sample.

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The devices according to the present invention may comprise a first element specifically adapted to measure the level of one or more protease enzymes, and one or more further elements specifically adapted to measure the level of one or more other protease enzymes. In certain embodiments, the apparatus or device(s) of the invention comprises a single element specifically adapted to measure the level of all analyte proteases, either separately or as a weighted average (weighted sum) in combination. For example, a single diagnostic device may be specifically adapted for detecting each of said proteases in said sample.

The term "specifically adapted" herein signifies that the device comprises at least one substance that reacts selectively with the protease analyte.

The device may comprise a selective binding partner such as an immunological binding partner, for the protease analyte and/or for a substrate peptide for the protease analyte, and/or for a cleavage fragment of said substrate peptide, and/or for a marker moiety attached to said substrate peptide. Suitable immunological binding partners include polyclonal antibodies and monoclonal antibodies.

If polyclonal antibodies are desired, a selected mammal, such as a mouse, rabbit, goat or horse, may be immunised with the monitored marker. The monitored marker used to immunise the animal can be obtained by any suitable technique, for example, it can be purified from a wound fluid sample from an infected wound, it can be derived by recombinant DNA technology or it can be synthesized chemically. If desired, the monitored marker can be conjugated to a carrier protein. Commonly used carriers to which the monitored markers may be chemically coupled include bovine serum albumin, thyroglobulin and keyhole limpet haemocyanin. The optionally coupled monitored marker is then used to immunise the animal. Serum from the immunised

animal is collected and treated according to known procedures, for example by immunoaffinity chromatography. Monoclonal antibodies to the monitored marker can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies using hybridoma technology is well known.

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- Panels of monoclonal antibodies produced against the monitored marker can be screened for various properties, *i.e.*, for isotype, epitope, affinity, etc. Alternatively, genes encoding the monoclonal antibodies of interest may be isolated from hybridomas, for instance by PCR techniques known in the art, and cloned and expressed in appropriate vectors. Chimeric antibodies, in which non-human variable regions are joined or fused to human constant regions may also be of use. Humanised antibodies may also be used. The term "humanised antibody", as used herein, refers to antibody molecules in which the CDR amino acids and selected other amino acids in the variable domains of the heavy and/or light chains of a non-human donor antibody have been substituted in place of the equivalent amino acids in a human antibody.
- 10 The humanised antibody thus closely resembles a human antibody but has the binding ability of the donor antibody. In a further alternative, the antibody may be a "bispecific" antibody, that is, an antibody having two different antigen binding domains, each domain being directed against a different epitope.
- 20 Phage display technology may be utilised to select genes which encode antibodies with binding activities towards the monitored marker either from repertoires of PCR amplified V-genes of lymphocytes from humans screened for possessing the relevant antibodies, or from naive libraries. The affinity of these antibodies can also be improved by chain shuffling.
- 25 Where antibodies generated by the above techniques, whether polyclonal or monoclonal, are employed as reagents in immunoassays, radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA), the antibodies can be labelled with an analytically-detectable reagent such as a radioisotope, a fluorescent molecule or an enzyme.
- 30 As used herein, the term "antibody" refers to intact molecules as well as to fragments thereof, such as Fab, F(ab')₂ and Fv, which are capable of binding to the antigenic determinant in question. Such antibodies thus bind to the monitored marker.

Suitably, the immunological or other binding partners are immobilised on a solid support material, for example by avidin-biotin linking, or dialdehyde derivatization of the support material, followed by cross-linking to a peptide binding partner. The apparatus and devices of
5 the invention may further comprise other immunological binding partners and/or reagents or indicator molecules may for example in a solution that is added to the wound fluid sample.

The solid support materials bearing immunological or other binding partners may be used in a range of immunoassays to analyse the presence of the analytes of interest. For example, the
10 support having antibodies or antibody fragments bound thereto may be used in sandwich immunoassay-type analysis. Alternatively, the support may have analog ligands bound to the antibodies, whereby the molecules present in the wound fluid are detected by affinity displacement immunoassay. Various other immunoassays will be apparent to persons skilled in the art.

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The analytes of interest are protease enzymes that can modify substrates such as proteins or polypeptides, by cleavage. Such modification of peptide substrates can be detected to determine the presence or absence of the analyte in a sample. In embodiments, the present invention provides a diagnostic device comprising an indicator moiety that is immobilized or
20 inhibited by a chemical moiety, wherein the chemical moiety comprises an exogenous peptide substrate for at least one of the analyte protease enzymes, and the exogenous peptide substrate is cleavable by the said at least one analyte protease enzymes to release or activate the indicator moiety.

25 Suitably, the indicator moiety comprises an indicator enzyme, an enzyme cofactor, a dye, a radioactive moiety, a spin label, a luminescent moiety or a fluorophore. Suitably, the indicator moiety comprises an indicator enzyme or a fluorophore. Suitable indicator enzymes may for example be selected from the group consisting of a laccase (CotA enzyme), alkaline phosphatase, p-galactosidase, acetylcholinesterase, green fluorescent proteins, luciferases and
30 horseradish peroxidases. Suitable fluorophores include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, coumarin derivatives such as 7-amino-4-methyl coumarin, and phycoerythrin. Suitable luminescent moieties include luminol, luciferase, luciferin, and aequorin.

In the devices in which the indicator moiety comprises an enzyme, the device suitably further comprises a substrate that interacts with the indicator enzyme to give a detectable spectrophotometric, colorimetric, fluorimetric, luminescent, electrochemical or radioactive signal.

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In certain embodiments, the indicator moiety comprises an indicator enzyme and the chemical moiety inhibits the indicator enzyme by sterically hindering an active site of the indicator enzyme, or by causing the indicator enzyme to fold into an inactive conformation. Alternatively or additionally, the chemical moiety may tether the enzyme to a solid substrate, whereby release from the substrate by action of the analyte protease activates the enzyme and/or allows the enzyme to migrate to a remote substrate location where it reacts with a suitable substrate (which may be immobilized at the remote location) to give a detectable signal. In yet other embodiments, the device comprises two indicator enzyme moieties linked by the chemical moiety, and cleavage of said peptide by the host-derived protease enzyme results in activation of both enzyme moieties.

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In other embodiments, the device comprises an indicator enzyme, and a cofactor for the enzyme that is immobilized or inhibited by the chemical moiety, whereby cleavage of peptide releases or activates the cofactor.

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A currently preferred indicator moiety comprises colloidal particles, suitably colloidal gold particles, conjugated to a peptide such as an antibody or a peptide substrate in known fashion. The colloidal particles are coloured, whereby accumulation of the colloidal particles in a test region or a control region of the device gives a visible colour the intensity of which increases with concentration of the particles, thereby allowing quantitative colorimetric determination of said concentration by colorimetry and/or comparison of the observed colour intensity with a reference colour to evaluate if the threshold has been exceeded.

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In certain embodiments, the indicator moiety is tethered to a solid substrate by said chemical moiety, and is released from said substrate by cleavage of said exogenous peptide substrate by said protease. In embodiments, the indicator moiety is conjugated to the exogenous peptide substrate, or to an immunological binding partner for the exogenous peptide substrate, and the protease level is determined by sandwich-immunoassay techniques involving binding of the exogenous peptide substrate to immunological binding partner for the exogenous peptide substrate. It will be appreciated that, in these embodiments, either the exogenous peptide

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substrate or the immunological binding partner for the exogenous peptide substrate may be anchored to a solid substrate.

One method for detecting the modification of a substrate by an enzyme is to label the substrate
5 with two different dyes, where one dye serves to quench the fluorescence of the other dye by fluorescence resonance energy transfer (FRET) when the dye molecules are in close proximity. A typical acceptor and donor pair for resonance energy transfer consists of 4-[[-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) and 5-[(2-aminoethylamino)naphthalene sulfonic acid (EDANS). EDANS is excited by illumination with a wavelength of
10 336 nanometers, and emits a photon with a wavelength of 490 nanometers. If a DABCYL moiety is located within 2 nanometers of the EDANS, this photon will be efficiently absorbed. DABCYL and EDANS can be attached to opposite ends of a peptide in the diagnostic material used in the systems of the present invention. If the peptide is intact, FRET will be very efficient. If the peptide has been cleaved by an enzyme analyte, the two dyes will no longer be
15 in close proximity and FRET will be inefficient. The cleavage reaction can be followed by observing either a decrease in DABCYL fluorescence or an increase in EDANS fluorescence (loss of quenching).

Another suitable diagnostic material for use in the systems of the present invention comprises a
20 chromogenic dye conjugated to a solid support by a suitable cleavable substrate moiety, such as a peptide. The chromogenic dye will change color when the linker group is cleaved by the enzyme of interest. For example, para-nitrophenyl is colorless when linked to the support, and turns yellow when cleaved. The analyte concentration can be determined by measuring absorbance at 415 nanometers. Other dyes that produce detectable color change upon cleavage
25 are known to those skilled in the art.

In yet another embodiment, the diagnostic material may comprise a colored support having a differently-colored molecule conjugated thereto by a linker moiety that can be cleaved by an analyte enzyme in the sample. Cleavage of the dye from the colored support can thereby result
30 in a color change of the diagnostic material.

The solid support materials used for the above identified assays of enzyme activity and immuno-assays may comprise any suitable natural or synthetic polymer, including insoluble polysaccharides such as cellulose or nitrocellulose, and synthetic polymers such as
35 polyacrylates. The cleavable cross-linkages, where present, generally comprise cleavable

oligopeptidic sequences or cleavable oligosaccharides, each typically of twenty residues or fewer, for example from 3 to 15 residues.

The sensitivity of the diagnostic material will depend on a number of factors, including the length of the cleavable linker sequences. Steric hindrance may also be reduced by coupling the cleavable oligopeptidic sequence to the polymer by means of an appropriate spacer. Thus, the oligopeptidic sequences may couple the polymers directly (in which case the cross-linkage consists of the oligopeptidic sequence) or by means of an appropriate spacer. Suitable conjugation methods incorporating spacers are described in US-A-5770229.

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Suitable chemical systems for use in the devices of the present invention are described in WO03/063693 and WO2005/021780, the entire contents of which are incorporated herein by reference.

15 In one embodiment, the indicator enzyme is a laccase that has been inhibited by the peptide substrate. Laccase (diphenol oxidase) is a member of the multi-copper oxidase family of enzymes. Generally, these enzymes require oxygen to oxidize phenols, polyphenols aromatic amines, and other non-phenolic substrates by one electron to create a radical species. It is a suitable indicator enzyme in part due to its stability and oxidation properties. The oxidation of
20 species results in an unpaired electron which generates a color change. CotA is highly thermostable.

CotA can be used in the apparatus and devices of the present invention by modifying the sequence to generate a proenzyme form. Analysis of the structure of CotA indicates that an
25 extension of suitable length appended onto the N-terminus of CotA can allow an appended inhibitor to be placed in the active site of the enzyme. The extension peptide is selected to be a cleavage target of the analyte protease. This will allow the blocking extension to be cleaved in the presence of the analyte protease. Analysis of the x-ray structure of CotA has shown that the length of the amino acid chain needed to reach the shortest distance around the structure is
30 about 3nm.

The modified enzymes with the peptide extension block can be prepared and screened for suitability using standard recombinant methods as described in more detail in WO2005/021780.

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As already noted, the endogenous proteases to be detected include human neutrophil elastase. For elastase, suitable substrate linkers may include one or more of the oligopeptidic sequences Lys-Gly-Ala-Ala-Ala-Lys-Ala-Ala-Ala- (SEQ ID NO: 1), Ala-Ala-Pro-Val (SEQ ID NO: 2), Ala-Ala-Pro-Leu (SEQ ID NO: 3), Ala-Ala-Pro-Phe (SEQ ID NO: 4), Ala-Ala-Pro-Ala (SEQ ID NO: 5) or Ala-Tyr-Leu-Val (SEQ ID NO: 6). For example, the substrate may be MeOSuc-Ala-Ala-Pro-Val-AMC (SEQ ID NO: 7), wherein MeOsuc is a succinyl methyl ester residue and AMC is a 7-amino-4-methyl coumarin residue.

The proteases to be detected also include matrix metalloproteinases, such as MMP-1 and MMP-9. Suitable cleavable linkers for matrix metalloproteinases may comprise the oligopeptidic sequence -Gly-Pro-Y-Gly-Pro-Z- (SEQ ID NO: 8), -Gly-Pro-Leu-Gly-Pro-Z- (SEQ ID NO: 9), -Gly-Pro-Ile-Gly-Pro-Z- (SEQ ID NO: 10), or -Ala-Pro-Gly-Leu-Z- (SEQ ID NO: 11), where Y and Z are amino acids.

Fragments and sequence variants of the polypeptides described above may also be used in the apparatus, devices and methods of the present invention. Functional variants can contain substitution of similar amino acids that result in no change or an insignificant change in function. Alternatively, such substitutions may positively or negatively affect function to some degree.

20

In one embodiment, the method, apparatus or device of the invention comprises a reference assay element for determining the total protein content of the sample, so that the measured levels of marker can be normalised to constant total protein level in order to increase accuracy.

In certain embodiments, the device according to the present invention comprises a housing containing one or more reagents and having an inlet provided therein for introduction of the sample. The housing may be at least partially transparent, or may have windows provided therein, for observation of an indicator region that undergoes a color or fluorescence change. In embodiments, the housing is in the form of a folding card. Suitably the folding card has two flaps joined by a hinge. A diagnostic strip is attached to the first flap. The second flap can fold over the first flap, and suitably a securing feature such as a retaining flap or adhesive strip is provided to secure the card in the folded-over configuration. A window is suitably provided in the first flap for observation of an indicator region of the test strip when second flap is folded over the first flap. A swab carrying a sample of wound fluid can be folded inside the card with the swab abutting an inlet region of the test strip. An opening may be provided in

the first flap above said inlet region of the test strip when the card is closed, so that one or more drops of elution fluid can be applied to the swab enclosed inside the folded card.

In certain embodiments, the device operates on the lateral flow principle. That is to say, said
5 device comprises a housing having an inlet for the sample and a liquid-permeable strip defining a fluid lateral flow path extending from the inlet. By "lateral flow", it is meant liquid flow in which the dissolved or dispersed components of the sample are carried, suitably at substantially equal rates, and with relatively unimpaired flow, laterally through the carrier. Suitably, the fluid flow path contains one or more porous carrier materials. The porous carrier
10 materials are suitably in fluid communication along substantially the whole fluid flow path so as to assist transfer of fluid along the path by capillary action. Suitably, the porous carrier materials are hydrophilic, but suitably they do not themselves absorb water. The porous carrier materials may function as solid substrates for attachment of reagents or indicator moieties. In certain embodiments of the present invention, the device further comprises a control moiety
15 located in a control zone in said device, wherein the control moiety can interact with a component of the wound fluid sample to improve the accuracy of the device.

The size and shape of the carrier are not critical and may vary. The carrier defines a lateral flow path. Suitably, the porous carrier is in the form of one or more elongate strips or columns.
20 In certain embodiments, the porous carrier is one or more elongate strips of sheet material, or a plurality of sheets making up in combination an elongate strip. One or more reaction zones and detection zones would then normally be spaced apart along the long axis of the strip. However, in some embodiments the porous carrier could, for example be in other sheet forms, such as a disk. In these cases the reaction zones and detection zones would normally be
25 arranged concentrically around the center of the sheet, with a sample application zone in the center of the sheet. In yet other embodiments, the carrier is formed of carrier beads, for example beads made from any of the materials described above. The beads may suitably be sized from about 1 micrometer to about 1mm. The beads may be packed into the flow path inside the housing, or may be captured or supported on a suitable porous substrate such as a
30 glass fiber pad.

As discussed above, the apparatus or device(s) according to the present invention are adapted to detect more than one protease. In one embodiment, a single device may be adapted to detect

each of the proteases to be determined. For example, a single device may be adapted to detect each of the elastase and matrix metalloproteinases to be determined. In any of these cases, the detection of more than one protease can be done by the use of several different reagents in a single reaction zone, or by the provision in a single device of a plurality of lateral flow paths
5 each adapted for detecting a different analyte. In certain embodiments, the plurality of lateral flow paths are defined as separate fluid flow paths in the housing, for example the plurality of lateral flow paths may be radially distributed around a sample receiving port. In some embodiments, the plurality of fluid flow paths are physically separated in a housing. In other embodiments multiple lateral flow paths (lanes) can be defined in a single lateral flow
10 membrane by depositing lines of wax or similar hydrophobic material between the lanes. In yet other embodiments, the substrate is reactive with more than one, or all, of the protease analyte enzymes to different degrees, whereby a single measurement gives a weighted average, also defined as an average protease activity weighted by said respective reactivities.

15 An absorbent element may suitably be included in these devices of the present invention. The absorbent element is a means for drawing the whole sample through the device by capillary attraction. The absorbent element is thus usually located at a downstream end of the flow path. Generally, the absorbent element will consist of a hydrophilic absorbent material such as a woven or nonwoven textile material, a filter paper or a glass fiber filter.

20

The apparatus or device(s) according to the present invention may further comprise at least one filtration element to remove impurities from the sample before the sample undergoes analysis. The filtration device may for example comprise a microporous filtration sheet for removal of cells and other particulate debris from the sample. The filtration device is typically provided
25 upstream of the sample application zone of the fluid flow path, for example in the inlet of the housing or in the housing upstream of the inlet.

In certain embodiments, the apparatus or devices according to the present invention include a control moiety in a control zone of the device, wherein the control moiety can interact with a
30 component of the wound fluid sample to improve the accuracy of the device. Suitably, the control zone is adapted to reduce false positive or false negative results. A false negative result could arise for various reasons, including (1) the sample is too dilute, or (2) the sample was too small to start with.

In order to address false negative mechanism, the control zone suitably further comprises a reference assay element for determining the total protease content or the total protein content of the sample, that is to say for establishing that the total protease content or the total protein content of the sample is higher than a predetermined minimum. It is possible to indicate the presence of protein by the use of tetrabromophenol blue, which changes from colorless to blue depending on the concentration of protein present. It is also possible to detect glucose (using glucose oxidase), blood (using diisopropyl-benzene dihydroperoxide and tetramethylbenzidine), leukocytes (using ester and diazonium salt). These may all be useful analytes for detection in the control zone for the reduction of false negatives.

Lateral flow devices that may be used with suitable modification are described, for example, in WO-A-2009024805, WO-A-2007128980, WO-A-2009063208 and WO-A-2006079826, the entire disclosures of which are incorporated herein by reference. The devices described in WO-A-2007128980 are especially suitable. These devices produce an output in the form of a colored line in an indicator region of the lateral flow substrate. The colour intensity of this line can be compared with a reference chart, or measured with a colorimeter, to determine if the threshold has been exceeded. It will be appreciated that other indicators could give a detectable fluorescence which can be compared with reference values to determine if the threshold has been exceeded.

Suitably, these devices are lateral flow devices wherein the sample is initially reacted with a protease analyte substrate (such as an exogenous peptide substrate) conjugated to a binding moiety, such as an immunological binding moiety or biotin. The sample is also mixed with a chromophore (such as colloidal gold) conjugated to a binding partner (such as an antibody) specific for the protease analyte substrate. In the absence of protease, the chromophore-binding partner conjugate binds to the substrate-binding moiety conjugate to form a complex containing both the binding moiety and the chromophore. In the presence of protease, the substrate is cleaved whereby little or no binding of the above components takes place. The mixture is introduced into a lateral flow strip having a sample line for selectively binding the binding moiety and a control line for selectively binding the binding partner. In the absence of protease, the above complex is bound at the sample line resulting in colour at the sample line. In the presence of protease, less of the above complex is formed and therefore less colour is

observed at the sample line. In both cases, chromophore-binding partner (present in excess in the starting mixture) is bound at the control line to give a colour indicative of a completed test.

In a further aspect, the present invention provides a diagnostic kit comprising a diagnostic
5 device according to any preceding aspect or embodiment, together with one or more of: a color chart for interpreting the output of the diagnostic device, a sampling device for collecting a sample of a biological fluid such as a wound fluid; a wash liquid for carrying a sample of fluid through the device, and a pretreatment solution containing a reagent for pretreatment of the fluid sample.

10

Where present, the sampling device may comprise a swab or a biopsy punch, for example a shaft having a swab or biopsy punch attached thereto. The swab may be any absorbent swab, for example a nonwoven fibrous swab. Typically the diameter of the swab is about 2 to about 5mm, for example about 3mm. In certain embodiments, the swab may be formed from a
15 medically acceptable open-celled foam, for example a polyurethane foam, since such foams have high absorbency and can readily be squeezed to expel absorbed fluids. The biopsy punch will typically be a stainless steel cylindrical punch of diameter about 1mm to about 10mm, for example about 3mm to about 8mm, suitably about 6mm.

20 The devices, kits and methods of the present invention are useful in prognosing or treating wounds that exude a wound fluid. Any type of wound may be diagnosed for treatment using the apparatus, device(s) and methods of the present invention. The wound is suitably a chronic wound. Suitably, the chronic wound is selected from the group consisting of venous ulcers, pressure sores, decubitus ulcers, diabetic ulcers and chronic ulcers of unknown aetiology.

25

When used herein, the term "combined amount" refers to a single numerical value that results from the application of a mathematical function to a plurality of values, for example those amounts obtained for a number of individual analytes. For example, the term "combined amount" may refer to the sum or product of a group of individual values. Typically, the term
30 "combined amount" relates to the sum of a group of individual values. For example, in suitable embodiments, the amount of elastase refers to elastase-like activity (*e.g.* in RFU/min/mL) and the amount of metalloproteinase (MMP) refers to total concentration of the respective analyte (*e.g.* in ng/mL).

When used herein, the term “quantifying” refers to measuring an absolute numerical quantity of a particular analyte(s) or substrate(s) in a sample, within the margins of experimental error.

The term “marker” or “analyte” refers to any chemical entity that is identified or determined
5 using the apparatus, devices, kits or methods defined herein. The markers or analytes determined or identified by the apparatus, devices, kits or methods of the present invention are protease enzymes.

The term “a wound fluid” refers to any wound exudate or other fluid (suitably substantially not
10 including blood) that is present at the surface of the wound, or that is removed from the wound surface by aspiration, absorption or washing. The determining, measuring or quantifying is suitably carried out on wound fluid that has been removed from the body of the patient, but can also be performed on wound fluid *in situ*. The term “wound fluid” does not normally refer to blood or tissue plasma remote from the wound site. The wound fluid is mammalian wound
15 fluid, suitably human wound fluid.

When used herein, the term “predetermined threshold” refers to a minimum level that the skilled person would determine is indicative of a non-healing wound based on statistical analysis of levels determined for known healing and non-healing wounds, for example as
20 explained further below. For the test to be clinically useful, the threshold should be set at an appropriate level so that non-healing wounds with high protease activity are correctly identified. Increasing the threshold will increase the chance of only non-healing wounds being over the threshold. However, if the threshold is too high, wounds that are non-healing due to a high level of proteases would not be identified and clinically this would mean they would not
25 receive the required protease modulating treatment.

When used herein, the term “control standard” or “control” refers to a data set or profile that can be used as a reference or comparison in order to define or normalise another data point or set of data. For instance, the term “control” or “control standard” may be data set or profile that is indicative of a particular sub-class of patient. In one embodiment, the control standard
30 is a data set or profile is a data set or profile indicative of healing or non-healing wound status.

When used herein, the term “simultaneously” when referring to determining or measuring a plurality markers and / or analytes refers to determining or measuring them substantially at the same time using a single apparatus or device. Alternatively, said determining or measuring
35 simultaneously may be performed using a plurality of apparatus or devices.

When used herein, the term “sequentially” when referring to determining or measuring plurality markers and / or analytes refers to determining or measuring them substantially in succession using a single apparatus or device. Alternatively, said determining or measuring
5 sequentially may be performed using a plurality of apparatus or devices.

In certain aspects or embodiments according to the present invention the term “determining” comprises establishing whether a weighted average (weighted sum) level of said elastase and one or more matrix metalloproteinase(s) exceeds a predetermined threshold value for said
10 weighted average. In other aspects or embodiments of the invention the term “determining” comprises measuring the level of elastase and establishing if said level exceeds a first predetermined threshold; and measuring the level of at least one matrix metalloproteinase and establishing if said level exceeds a second predetermined threshold. In certain aspects or
15 embodiments thereof the term “determining” includes measuring a numerical value of said proteases; for example “determining” includes measuring the activity and or concentration of one or more analytes in a wound sample or a weighted average thereof.

The term “comprising” encompasses “including” as well as “consisting of” e.g. an apparatus “comprising” X may consist exclusively of X or may include something additional e.g. X + Y.
20

The word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

25 The term “about” in relation to a numerical value x is optional and means, for example, $x \pm 10\%$.

The present invention enables a care giver or a patient to determine quickly and reliably whether a wound is likely to be non-healing, and to select an appropriate therapy based on this
30 determination. For example, non-healing wounds may require the application of special wound dressings such as wound dressings comprising oxidized cellulose, to promote healing. Accordingly, in a further aspect, the present invention provides a method of treatment comprising determining whether a wound is healing or non-healing in accordance with the

invention, followed by applying an oxidized cellulose containing wound dressing to the wound if it is non-healing or a different kind of dressing if it is healing.

The term "oxidized cellulose" refers to any material produced by the oxidation of cellulose.

- 5 In suitable embodiments of the invention, the wound dressing comprises oxidized cellulose. In one embodiment, the oxidized cellulose dressing comprises oxidized regenerated cellulose. Typically, the wound dressing further comprises collagen or chitosan.

In particular embodiments, the oxidized cellulose in the wound dressing material is complexed
10 with collagen and/or chitosan to form structures of the kind described in WO 98/00180, EP-A-1153622, WO 2004/026200, EP-A-1539258, WO 2004/024197 and/or EP-A-1536845, the entire contents of which are expressly incorporated herein by reference. For example, the oxidized cellulose may be in the form of milled ORC fibres that are dispersed in a freeze-dried collagen or chitosan sponge. This provides for sustained release of the oxidized cellulose to
15 the wound, together with certain therapeutic and synergistic effects arising from the complexation with collagen. Suitably, the weight ratio of oxidized cellulose to collagen and/or chitosan in the wound contacting material is from about 10:1 to about 1:10, for example from about 70:30 to about 30:70. Suitably, the wound contacting material comprises at least 75% on a dry weight basis of oxidized cellulose, collagen and chitosan, more suitably at least 90% and
20 most suitably it consists essentially of oxidized cellulose, collagen and/or chitosan. Such oxidised cellulose wound dressings may also comprise silver. Suitable commercially available wound dressings comprising oxidized cellulose are PROMOGRANTM and PROMOGRAN PRISMATM (Systagenix Wound Management).

25 DESCRIPTION OF THE FIGURES

Embodiments of the invention will now be described further, with reference to the accompanying drawings, in which:

Fig. 1 shows a box plot of measured percentage reduction in wound area after 2 or 4 weeks for healing versus non-healing diabetic foot ulcers;

- 30 **Fig. 2** shows a box plot of measured percentage reduction in wound area after 2 or 4 weeks for healing versus non-healing leg ulcers;

Fig. 3 shows a box plot of measured percentage reduction in wound area after 2 or 4 weeks for healing versus non-healing pressure ulcers;

Fig. 4 shows a graph of measured hNE versus MMP activity in healing wounds;

- 35 **Fig. 5** shows a graph of measured hNE versus MMP activity in non-healing wounds;

Fig. 6 shows a graph of the probability of a wound being non-healing versus measured hNE activity for a population of healing and non-healing wounds;

Fig. 7 shows a graph of the probability of a wound being non-healing versus measured MMP activity for a population of healing and non-healing wounds;

5 Fig. 8 shows a folding card device according to the present invention with the card in the open configuration;

Fig. 9 shows a detail of the sample receiving well in the device of Fig. 8;

Fig. 10 shows a front view of the folding card device of Fig. 8 in the closed position;

Fig. 11 shows a schematic view of the test reagents present in the sample receiving well of Fig.

10 9;

Fig. 12 shows a schematic view of the lateral flow test strip in the device of Fig. 8;

Figs. 13(a) and 13(b) show a schematic of the interaction between the sample, the test strip, and the test reagents for a sample containing low levels of protease analyte;

Figs. 14(a) and 14(b) show a schematic of the interaction between the sample, the test strip,

15 and the test reagents for a sample containing high levels of protease analyte;

Fig. 15 shows a schematic plan and side view of the lateral flow test strip of the device of Fig. 8.

DETAILED DESCRIPTION OF THE INVENTION

20 Referring to Figs. 8-10, the folding card device 2 according to the present invention comprises front and back flaps 3,4 of cartonboard material joined by a living hinge 5. A strip 6 of pressure sensitive adhesive on the back flap 4 provides a retaining element for retaining edge 7 of the front flap when the card device is folded shut. The adhesive strip 6 is covered by a releasable cover sheet (not shown) that is removed immediately before use. A lateral flow test

25 strip 8 is mounted on the inside surface of the front flap 3, for example by adhesive. A thermoformed plastic swab well 10 is mounted in the back flap 4 and partially covered by a cover sheet 12 having apertures 14,16 located respectively above upper and lower regions of the plastic swab well 10. The swab well 10 contains dry reagents 11 as described further below. The aperture 16 is located such that, when the card is folded shut, the aperture 16 abuts

30 a sample receiving region 13 of the test strip 8, but aperture 14 does not abut the test strip 8.

A window 9 in the front flap 3 is located over a region of the test strip where the sample and control read-out lines are located, so that this region of the test strip can be viewed when the card is folded shut. One or more reference colour areas 15 may be printed on the outside of the

35 front flap 3 for comparison with a colour displayed in the read-out lines.

In use, the swab well 10 is primed by the addition of a few drops of a wash liquid, for example a saline solution. A swab having a sample of wound fluid is inserted into the swab well 10 through the aperture 14 and rotated to release the sample into the wash liquid in the well. The card is then folded shut and secured shut with the adhesive strip 6. The swab is usually attached to a swab shaft or stick 17, which can project out of the bottom of the closed card device. When the card is shut, the liquid containing the sample passes through the aperture 16 by capillary action into a sample receiving region 13 of the test strip 8. The user may be instructed to allow the reagents in the swab well to react with the sample for a predetermined time, e.g. 10 minutes, before closing the card.

Referring to Figs. 11-14, lateral flow test chemistry similar to that described in WO2007/128980 is used in this embodiment. The swab well 10 contains colloidal gold particles 20 conjugated to antibody 22 that is selective for a binding partner that is immobilized on the control line of the test strip as explained further below. The colloidal gold particles are sized such that they exhibit a red coloration when dispersed in a liquid or solid matrix. The swab well 10 further comprises the peptide substrate 24 for the analyte protease(s) conjugated to biotin 26.

Referring to Fig. 15, the test strip 8 comprises a sample receiving area 28 of blotting paper or similar absorbent sheet material, a porous area 29 impregnated with colloidal gold 20 conjugated to an antibody specific for the peptide substrate 24, a test area 30, and an absorbent reservoir area 32 for drawing the liquid through the test area 30 by capillary action. The test area comprises a sample read-out band 31 in which the test area substrate is conjugated to polystreptavidin 34, and a control read-out band 33 in which the test area substrate is conjugated to an antigen 35 that binds strongly to the antibody 22 on the colloidal gold particles from the swab well.

Referring to Fig. 13, the operation of the device when the sample does not contain protease analyte is shown. In the absence of protease analyte in the sample, the cleavable peptide moiety 24 binds strongly to the conjugate antibody on the colloidal gold particles of the test strip, thereby forming a complex comprising the colloidal gold 20 joined to the biotin 26. This complex binds to the streptavidin in the sample read-out band, thereby producing a red colour in this band due to the presence of the colloidal gold. The other gold conjugate from the swab well binds to the antigen in the control read-out band of the strip, thereby producing a red

colour in this band due to the presence of the colloidal gold. Therefore, a red line in both the sample band and the control band signifies that the test has been performed successfully, but that the amount of analyte protease in the sample is below the threshold used to define a 90% probability of non-healing wounds.

5

Referring to Fig. 14, the operation of the device when the sample contains protease analyte is shown. The addition of the protease analyte to the swab well 10 causes the cleavable peptide moiety 24 to be cleaved by the protease analyte. The cleavage fragments do not bind strongly to the conjugate antibody on the colloidal gold particles of the test strip, and therefore binding of the colloidal gold 20 to the biotin 26 does not take place. As a result, little or none of the gold binds to the streptavidin in the sample read-out band, thereby producing little or no red colour in this band due to the presence of the colloidal gold. The other gold conjugate from the swab well still binds to the antigen in the control read-out band, thereby producing a red colour in this band due to the presence of the colloidal gold. Therefore, a red line in only the control band signifies that the test has been performed successfully, and that the amount of analyte protease in the sample is above the threshold characteristic of non-healing wounds. This is as an indication of a non-healing wound.

EXAMPLES

20 *Clinical study and patient selection*

All patients enrolled in this study had chronic wounds of at least 30 days duration and a surface area of at least 1 cm². Exclusion criteria included concomitant conditions or treatments that may have interfered with wound healing and a history of non-compliance that would make it unlikely that a patient would complete the study.

25

Patients meeting the patient selection criteria were enrolled, and wound fluid collected. Informed consent was obtained from all patients or their authorised representatives prior to study enrolment and the protocol was approved by the Ethics Committee at the participating study centre prior to the commencement of the study. The studies herein were conducted in accordance with both the Declaration of Helsinki and Good Clinical Practice.

30

Determination of healing status

Wherever possible, the wound size was measured and recorded on the day that the swab was taken. The clinicians retrospectively recorded the wound size at Week 2 (2 weeks prior to the swab being taken) and Week 4 (4 weeks prior to the swab being taken) using the patient

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records. In some cases either Week 4 and / or Week 2 was not available, due to various reasons such as the patient being new to the clinic. The criteria used to classify wounds as healing or non-healing for each ulcer type is described below.

- 5 Diabetic foot ulcers were classed as healing if they reached at least 50 % reduction by Week 4. Patients that did not meet this criterion by 4 weeks were classed as non-healing. If Week 4 was not available then the wound size at Week 2 was utilised to classify the healing status; if the wound size had increased the patient was classed as non-healing, if the wound had met the 50% reduction within the 2 week timeframe then it was classed as healing, if the wounds had
10 decreased but not met the 50% threshold by 2 weeks this was classed as unknown healing status.

- The venous leg ulcers and pressure ulcers were classed as healing if they reached at least 30 % reduction by Week 4. Patients that did not meet this criterion by 4 weeks were classed as non-
15 healing. If Week 4 was not available then the wound size at Week 2 was utilised to classify the healing status; if the wound size had increased the patient was designated as non-healing, if the wound had met the 30% reduction in 2 weeks then this was classed as healing, if the wounds had decreased but not met the 30% threshold by 2 weeks this was classed as unknown healing status.

20

- A summary of the healing data for each wound type is shown in Tables 1-3 below, and graphically in Figs 1-3. Results are displayed as percentage decrease in wound size from either Week 4 or Week 2. The data is displayed for each wound type to show the distribution of patients in each group. The maximum increase that has been recorded in this data is an
25 increase in wound size of 200%; therefore any values over this will be classed as a 200% increase. This is so that the data can be clearly viewed on the graphs and the data is not skewed by a few very high values.

Table 1: Healing and non-healing diabetic foot ulcers.

	Healing (n=13)	Non-healing (n=18)
Range	51 to 95 (wound size decrease 51 to 95%)	-200 to 45 (200% increase to 45% decrease in wound size)
Median % reduction	67 (67% decrease in wound size)	-10 (10% increase in wound size)
Mean % reduction	69 (69% decrease in wound size)	-38 (38% increase in wound size)

Standard Deviation	14.3	80.6
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Table 2: Healing and non-healing leg ulcers.

	Healing (n=20)	Non-healing (n=32)
Range	36 to 98 (Reduction in wound area of 36 to 98%)	-200 to 28 (200% increase in wound area to 28% decrease)
Median % reduction	65 (65% reduction in wound area)	-9 (9% increase in wound area)
Mean % reduction	66 (66% reduction in wound area)	-22 (22% increase in wound area)
Standard Deviation	18.9	48.1

Table 3: Healing and non-healing pressure ulcers.

	Healing (n=3)	Non-healing (n=7)
Range	31 to 77 (Decrease of 31% to 77%)	-100 to 23 (100% increase to 23% decrease)
Median % reduction	55 (55% decrease in wound size)	5 (5% decrease in wound size)
Mean % reduction	54 (54% decrease in wound size)	-8 (8% increase in wound size)
Standard Deviation	43.0	23.0

5

Wound fluid collection

For each subject enrolled, two swabs were collected from the wound. Prior to swabbing, the wound was cleansed with sterile saline to remove all loose debris, remains of therapeutic agents (e.g. enzymatic debriders, gels, dressings, etc.) and necrotic tissue. No sharp wound debridement was performed prior to sample collection, and hemostasis was completed before obtaining the specimen. The wound area to be swabbed was moistened with a few (up to five) drops of saline. Swabbing was performed on areas free from blood, necrotic material or thick slough.

15

Protein Assay

Total protein present in each extracted wound fluid sample was determined using the Bradford protein assay. The protein binding solution comprises 1 ml Coomassie Brilliant Blue stock solution 200mg-Coomassie Brilliant Blue G250, Sigma Chemical Co., dissolved in 50 ml ethanol-90%); 2ml orthophosphoric acid (85% w/v); in a final volume of 20 ml with distilled water. This solution was filtered (Whatman #1 filter paper) and used immediately. The protein level in a sample wound fluid was measured by mixing 10- μ l sample or standard with

20

190- μ l of the protein binding solution in a microtitre well and incubating for 30mins at ambient temperature prior to reading absorbance at 595nm. The concentration of protein was estimated from a standard calibration of BSA (bovine serum albumin prepared in distilled water; Sigma Chemical Co.) ranging from 1.0 to 0.01 mg/ml.

5

Measurement of Human Neutrophil Elastase Activity

hNE activity was measured spectrophotometrically using a substrate which mimics the cleavage site of the enzyme. The substrate sequence used was specific for neutrophil derived elastase; MeOSuc-Alanine-Alanine-Proline-Valine- 7 amino 4 methyl coumarin (Bachem, Switzerland) (SEQ ID NO: 7). This short peptide substrate also contains the fluorescent reporter group, 7-amino 4-methyl coumarin, which is released upon substrate hydrolysis. Enzyme activity was then calculated by measuring the rate of production of the fluorimetric compound at 450nm (excitation 380nm). Activity was expressed as relative fluorescence units per minute and converted to milliunits of elastase activity per minute per 110 μ L swab extract (mU/min/110 μ L) from a standard curve.

The substrate, MeOSuc-Alanine-Alanine-Proline-Valine- 7 amino 4 methyl coumarin (SEQ ID NO: 7) shows good specificity to human elastase. *Pseudomonas aeruginosa* elastase is not able to cleave the peptide, demonstrating that there is no cross reactivity with the bacterial elastase. Although the substrate is most susceptible to cleavage by elastase, it can also be cleaved to a much lesser extent by Proteinase 3, a neutrophilic protease with similar substrate specificity to Elastase.

Measurement of Matrix Metalloproteinase (MMP) activity

Matrix metalloproteinase (MMP) activity was measured spectrophotometrically using a substrate which mimics the cleavage site of the enzyme. The substrate sequence used was specific for MMP activity; Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂-TFA [Mca=(7-methoxycoumarin-4-yl)acetyl; Dpa=N-3-(2,4-dinitrophenyl)-L- α,β -diaminopropionyl] (Enzo Life Sciences) (SEQ ID NO: 12). This peptide substrate contains the fluorescent reporter group 7-methoxycoumarin-4-yl) acetyl, which is released upon substrate hydrolysis. Enzyme activity was then calculated by measuring the rate of production of the fluorimetric compound at 400 nm (excitation 328 nm). Activity was expressed as relative fluorescence units per minute and converted to units of MMP activity per minute per 110 μ L swab extract (U/min/110 μ L) from a standard curve.

35

Data analysis

A total of 93 samples were analysed using Microsoft Excel and Minitab 16 in order to:

- (a) Review the distributions of quantitative data and consider possible data transformations;
- (b) Examine interactions between measured levels of the specific protease;
- 5 (c) Obtain graphical representation of potential regression between Healing and Non-healing quantitative protease measured data; and
- (d) Review the application of Binary Logistical Regression as a potential model.

- All four data sets [(hNE, healing) (hNE, non-healing) (MMP, healing) (MMP, non-healing)]
- 10 have a large negative skew, and given the number of observations in each data set ($n = 36$ $n = 57$ * $n = 36$ $n = 57$ * respectively) then an assumption of normality cannot be made. [* on the advice of researchers one data point was omitted as being invalid as sample was taken earlier than standard period]. Standard transformations had marginal affect on degree of skewness and more extreme approaches were delayed until further evaluations made this to be considered
- 15 necessary.

Scatter graphs of hNE vs MMP at both categories showed limited evidence for a correlation between the two parameters (see Figs. 4 and 5) and non-correlation has been assumed.

Scatter diagrams for both protease measurements with category showed an overlapping distribution and hence a potential regression fit using a logistical approach was considered.

- 20 Using Minitab 16 and Stats/regression/binary logistical a logistical model was evaluated. High leverage points on hNE ($n=1$) and MMP ($n=2$) were omitted from the models before reanalysis. Diagnostic indicators showed no reason to discount the model and without a further detail assessment or data transformation an expected distribution was obtained for both hNE and MMP measurement. This is summarised below.

25 hNE Activity

Reported Result mU/100u	Estimated Probability of Non-healing Wound	Standard Error	CI (95%)
17	0.80	0.08	(0.62,0.93)
25	0.90	0.08	(0.66,0.98)
33	0.95	0.05	(0.70,0.99)
55	0.99	0.02	(0.78,1.0)

MMP Activity

Reported Result U/100u	Estimated Probability of Non-healing Wound	Standard Error	CI (95%)
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32	0.80	0.08	(0.61,0.92)
48	0.90	0.07	(0.65,0.98)
64	0.95	0.05	(0.69,0.99)
100	0.99	0.02	(0.76,1.0)

The statistical report concluded that there was limited evidence for any correlation between the two reference assay results. Figure 4 shows a scatter diagram of protease activity in healing and non-healing wounds, some wounds have elevated levels of MMP activity but low levels of hNE activity, and vice versa. This may be due to the fact that several proteases can degrade the same substrates, so they may be able to compensate for each other, also, different wounds may have different profiles of elevated protease activity depending on the stage of the inflammatory response. Therefore the reference assays have been given individual thresholds, so if the protease activity is above either the hNE activity or the MMP activity threshold it will be classed as having elevated protease activity.

The results obtained from the application of a Binary Logistical Regression model provided an estimate of the probability of a wound being Non-healing for any given value of measured Protease activity levels. The Binary Logistical model can be used to provide a probabilistic estimate of the categorisation of the wound (healing or non-healing) based upon measured levels of protease enzyme activity obtained from wound fluids. The model can be used to calculate the thresholds of hNE activity and MMP activity that would be required for any given probability that is applied to the model. We have applied a 0.90 estimated probability of non-healing, this sets the thresholds at a value of 25 mU/110µL for hNE activity and 48 U/110µL for MMP activity. That means that there is a 90 % probability that any sample which is over 25 mU/110 µL hNE and / or 48 U/110µL MMP is a non-healing wound with elevated protease activity.

Based on the data 12.9 % of wounds would be over these thresholds and the positive predictive value (PPV) for non-healing wounds is 83.3%. Figures 6 and 7 shows the hNE and MMP activity versus cumulative probability of non-healing wounds.

CONCLUSION

In summary, the present inventors have discovered that non-healing wounds can be distinguished from healing wounds by determining a weighted average (weighted sum) of the levels of human neutrophil elastase (hNE) and at least one matrix metalloproteinase (MMP), or, more suitably two or more matrix metalloproteinases (MMP); and assigning said wound to

a non-healing category if said weighted average exceeds a threshold level. The measurement of both analyte types compensates for inter-patient variations in the individual analytes and thereby reduces false negative results. The selection of a sufficiently high threshold reduces false positive results. Overall at least 80% or 90% of the wounds giving a positive result in this
5 test are found to be non-healing

The above embodiments have been described by way of example only. Many other embodiments falling within the scope of the accompanying claims will be apparent to the skilled reader.

CLAIMS

1. A method of wound prognosis comprising the steps of: determining whether a
5 weighted average level of human neutrophil elastase (hNE) and one or more matrix
metalloproteinase (MMP) in a sample of wound fluid from a wound exceeds a predetermined
threshold value for said weighted average; and assigning said wound to a non-healing category
if said weighted average exceeds said predetermined threshold value for said weighted
average.
- 10 2. A method according to claim 1, wherein the one or more matrix metalloproteinases are
selected from MMP-1, MMP-2, MMP-8, MMP-9, MMP-12, MMP-13, combinations thereof,
and total MMP.
- 15 3. A method according to claim 2, wherein the one or more matrix metalloproteinases
comprise MMP-1 and MMP-9.
4. A method according to any preceding claim, wherein said step of determining is
performed on a sample of wound fluid that has been removed from the body.
- 20 5. A method according to any preceding claim, wherein said step of determining
comprises allowing the analyte proteases to cleave a peptide substrate, whereby said level
corresponds to an activity of the analyte proteases.
- 25 6. A method according to any preceding claim, further comprising determining said
threshold by the steps of:
determining from measurements of wound area versus time which wounds in a
population of chronic wounds are healing and which are non-healing;
determining the weighted average level of hNE and one or more MMP in wound fluid
30 samples taken from each of the healing and non-healing wounds; and
selecting said threshold such that the threshold identifies the largest number of non-
healing wounds provided that at least about 80% of the wounds falling above said threshold is
non-healing.

7. A method according to claim 6, wherein said population comprises at least about 30 healing wounds and at least about 30 non-healing wounds.
8. A method according to claim 6, wherein at least about 90% of the wounds falling
5 above said threshold is non-healing.
9. A method of wound treatment comprising the steps of: diagnosing a wound by a method according to any of claim 1 to 8, followed by applying a wound dressing comprising oxidized cellulose to the wound if it is in the non-healing category.
- 10
10. A method according to claim 9, wherein the wound dressing comprises a combination of oxidized regenerated cellulose with collagen and/or chitosan in the dry weight ratio of from about 10:1 to about 1:10.
11. A device for simultaneously or sequentially measuring the levels of hNE and of at least one MMP in a sample of wound fluid, and for providing a detectable output if a weighted average level of said hNE and at least one MMP exceeds a predetermined threshold.
12. A device according to claim 11, wherein the device comprises an immunological
20 binding partner for said hNE and/or for said at least one MMP and/or for an exogenous peptide substrate of said hNE or MMP.
13. A device according to claim 11, wherein the device comprises an indicator moiety that is immobilized or inhibited by a chemical moiety, wherein said chemical moiety comprises an
25 exogenous peptide substrate for said hNE and/or said MMP, and said exogenous peptide substrate is cleavable by said hNE and/or said MMP to release or activate said indicator moiety.
14. A device according to claim 13, which is a lateral flow device comprising:
30 a strip of porous material defining a fluid flow path having a sample application zone at one end of said strip;
an indicator moiety that is bound to a solid substrate by means of a peptide linker moiety that is cleavable by said hNE and/or said one or more MMPs, said solid substrate being located in a reaction zone of said fluid flow path;

a detector moiety located in a detection zone downstream from the reaction zone in said fluid flow path, wherein the detector moiety interacts with an indicator moiety that has been cleaved from said solid substrate to produce a detectable change in said detection zone.

- 5 15. A device according to claim 13, which is a lateral flow device comprising:
an exogenous peptide substrate for said hNE and/or one or more MMPs conjugated to a binding moiety;
a chromophore conjugated to an immunological binding partner specific for said exogenous peptide substrate; and
10 a solid substrate in the form of lateral flow strip, wherein a sample line region of said solid substrate has bound thereto a first binding partner for binding the binding moiety to immobilize the binding moiety.

16. A device according to any of claims 13 to 15, further comprising a control moiety
15 immobilized in a control zone in said device, wherein the control moiety can interact with a component of the wound fluid sample to give an indication of a completed test.

17. A device according to any of claims 13 to 16, wherein the device comprises a reference assay element for establishing that the total protease content or the total protein content of the
20 sample is higher than a predetermined minimum.

18. A device according to any of claims 13 to 17, wherein the device comprises a plurality of fluid flow paths each for detecting a different protease analyte enzyme.

- 25 19. A device according to any of claims 13 to 18, wherein said device comprises a folding card having a front flap and a back flap joined by a hinge, and said lateral flow substrate is mounted on an inside surface of said folding card.

20. A device according to claim 19, further comprising a sample receiving well on an
30 inside surface of the folding card opposite said inside surface having said lateral flow substrate.

21. A device according to any of claims 11 to 20, for use in a method according to any of claims 1 to 8.

FIG. 1
Boxplot of Percent Reduction in Healing and Non Healing Diabetic Foot Ulcers

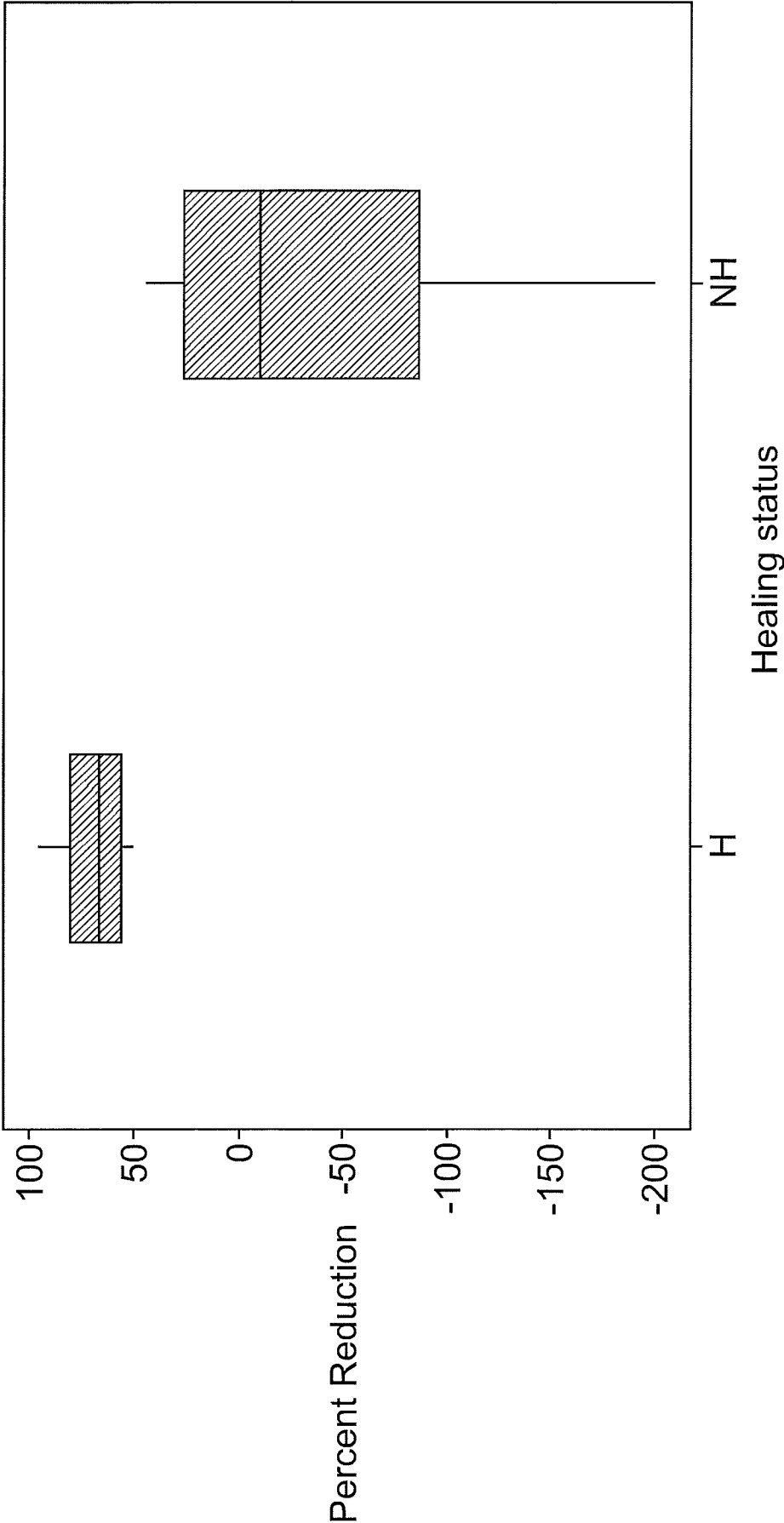


FIG. 2
Boxplot of Percent Reduction in Healing and Non Healing Leg Ulcers

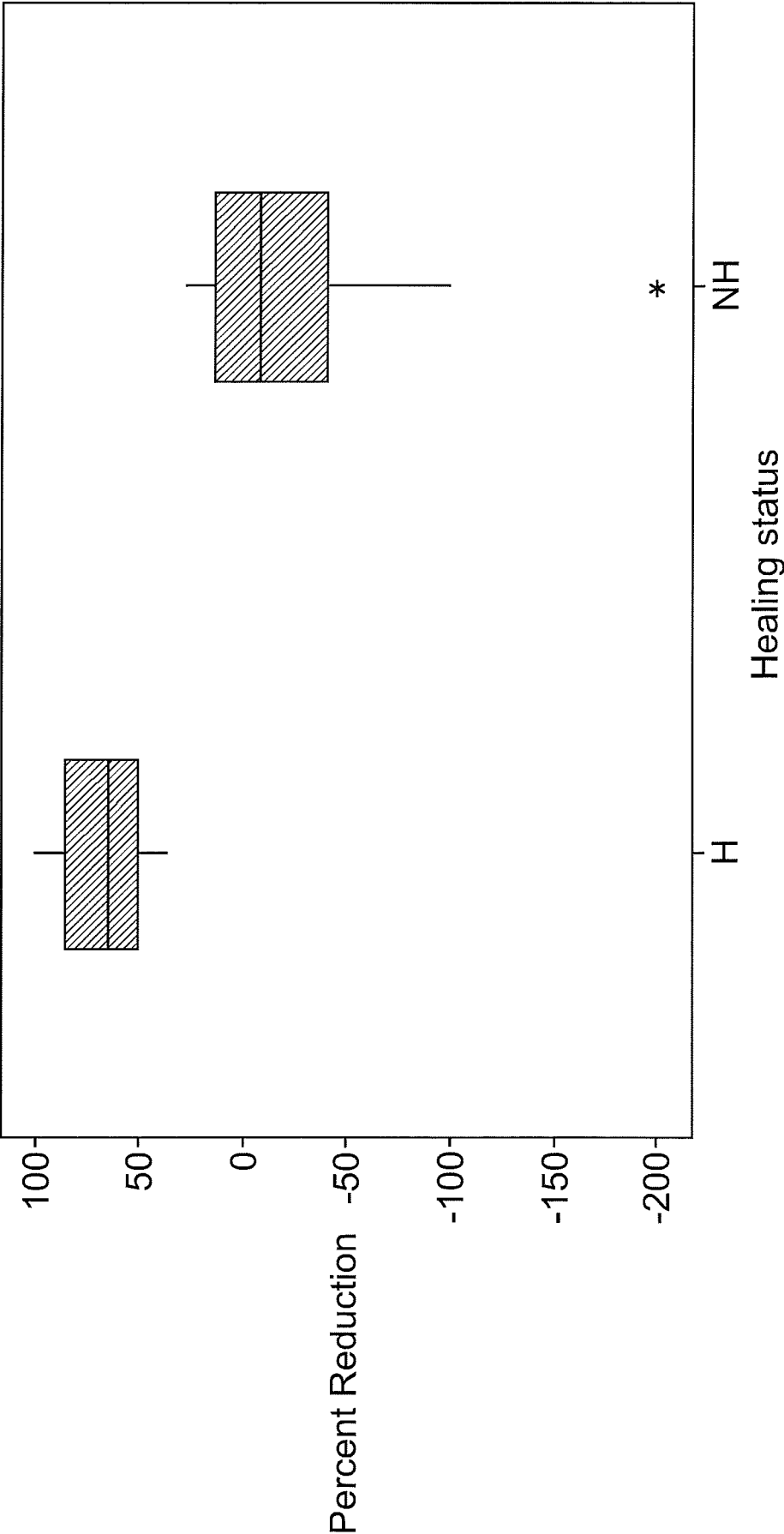


FIG. 3
Boxplot of Percent Reduction in Healing and Non Healing Pressure Ulcers

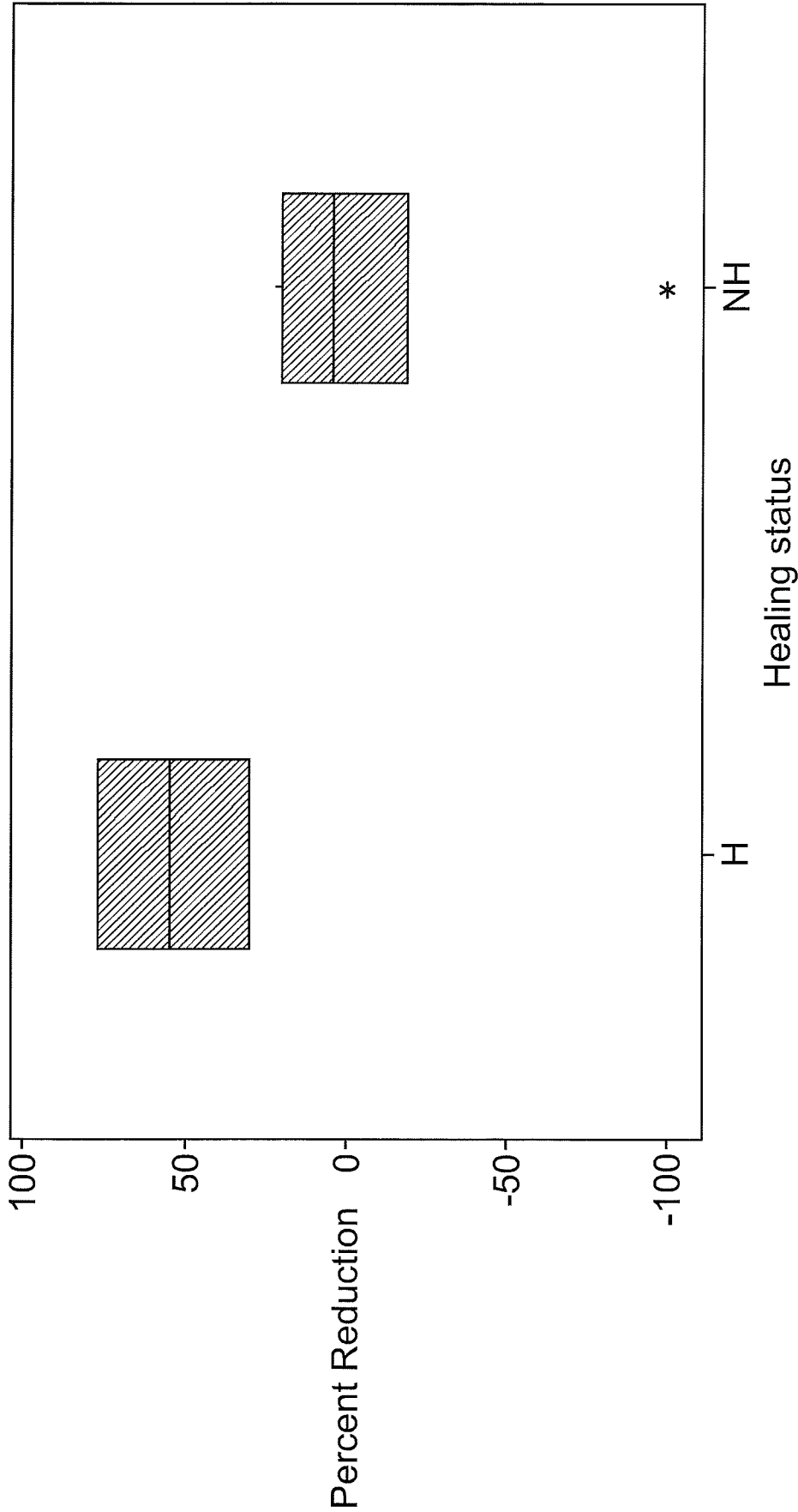


FIG. 4
MMP vs HNE Activity Healing Wounds

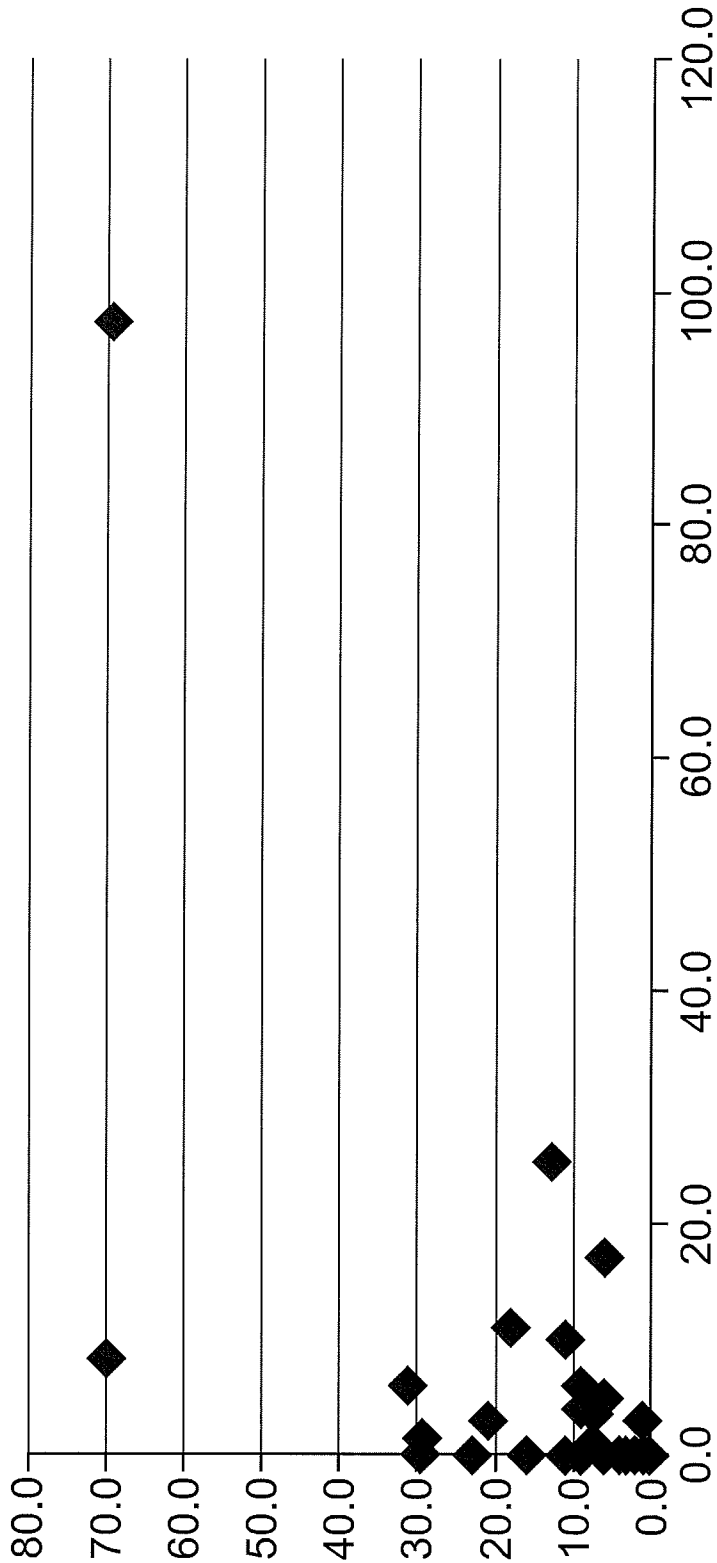
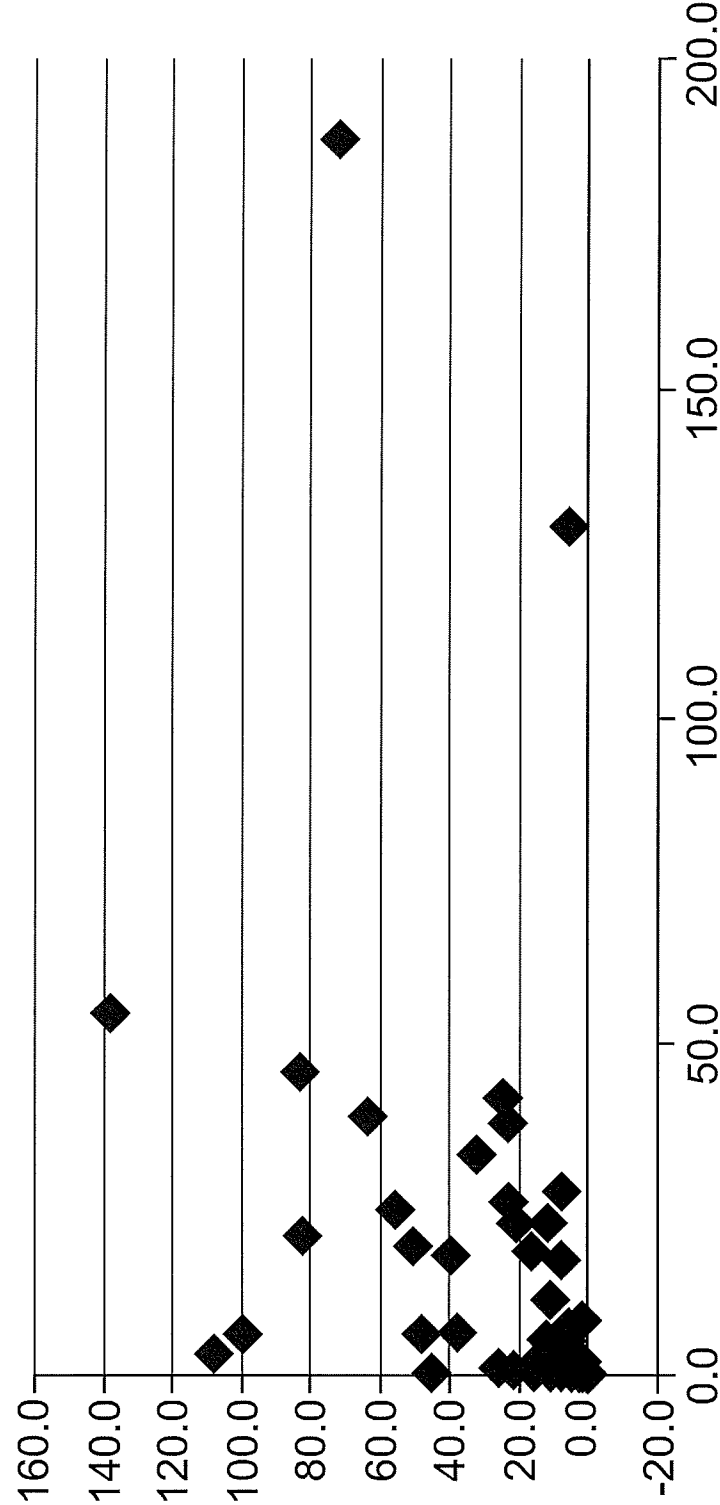
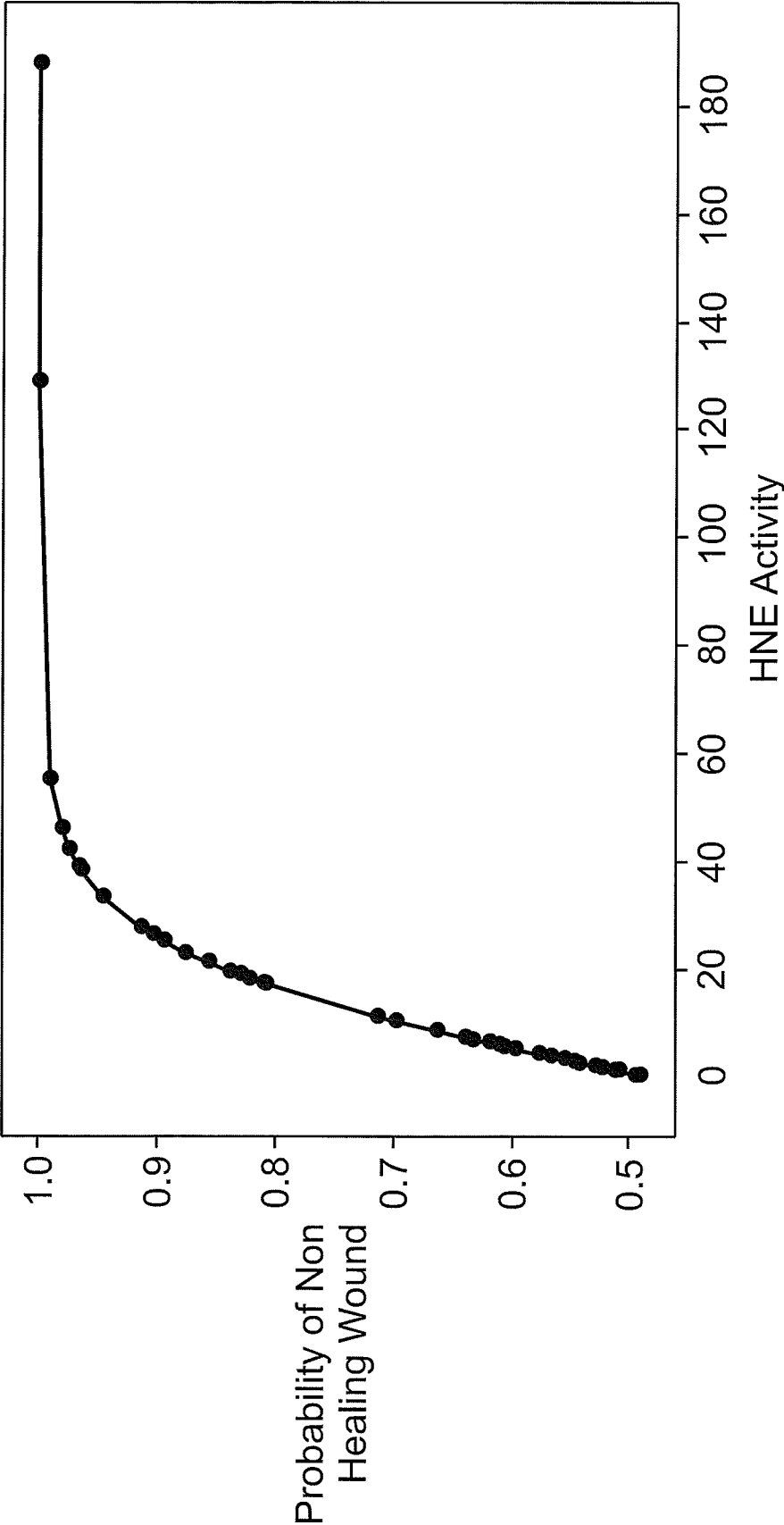


FIG. 5
MMP vs HNE Activity Non Healing Wounds



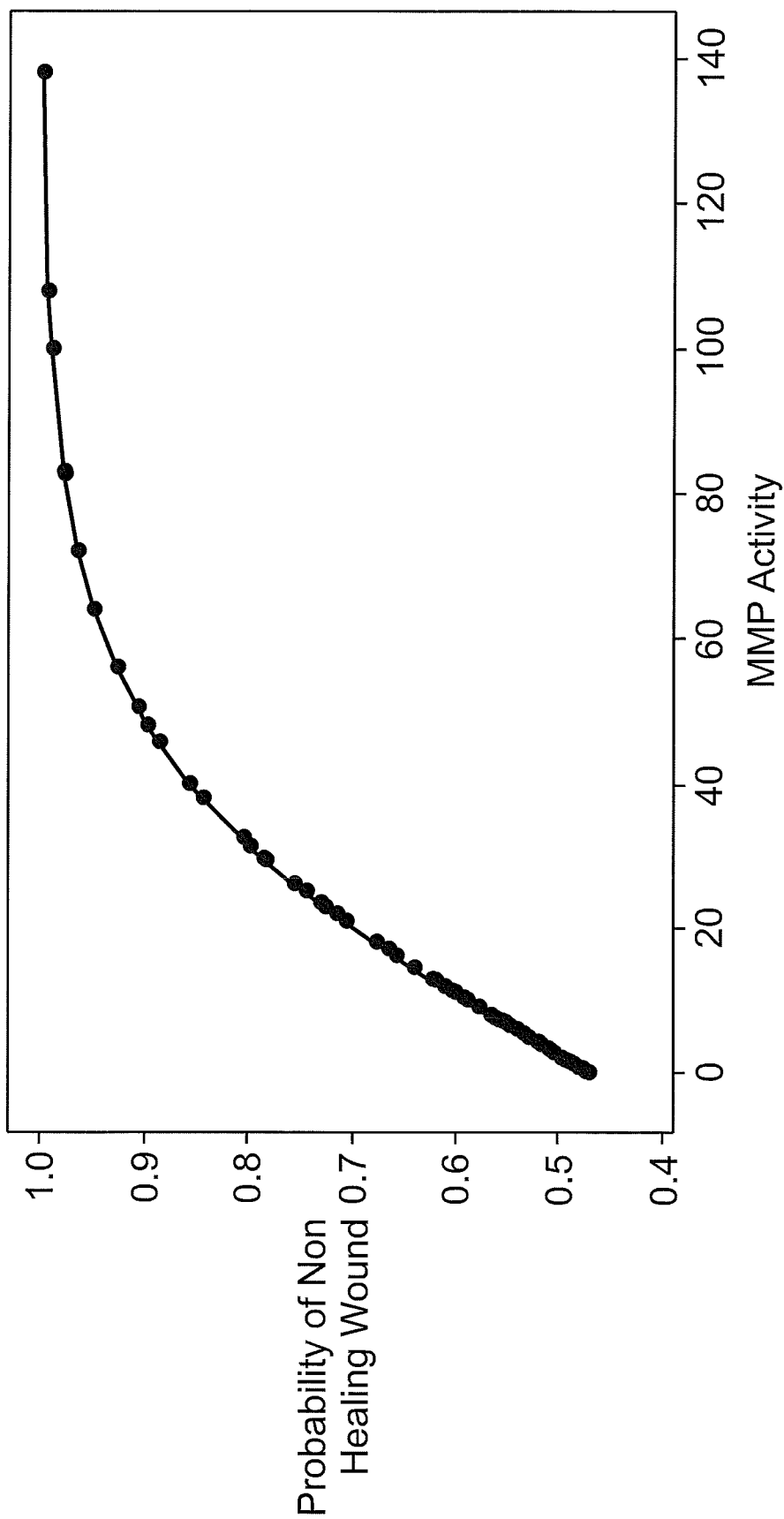
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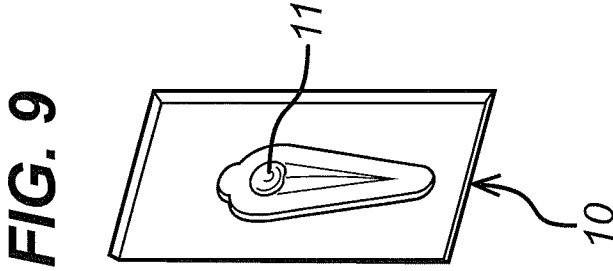
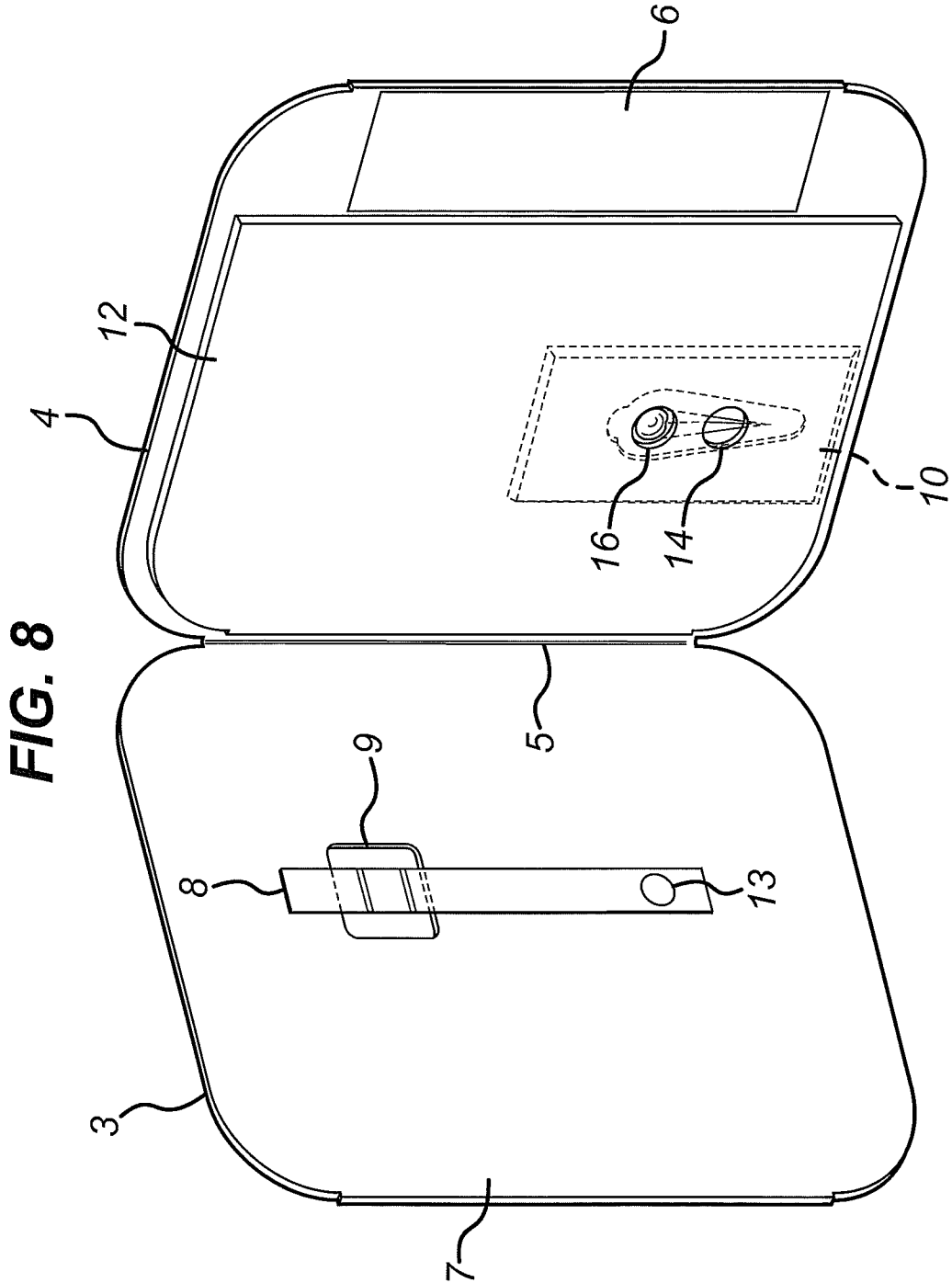
FIG. 6
Probability of Non Healing Wound vs Protease HNE Activity mU / 100u



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FIG. 7
Probability of Non Healing Wound vs Protease MMP Activity U / 100u





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FIG. 10

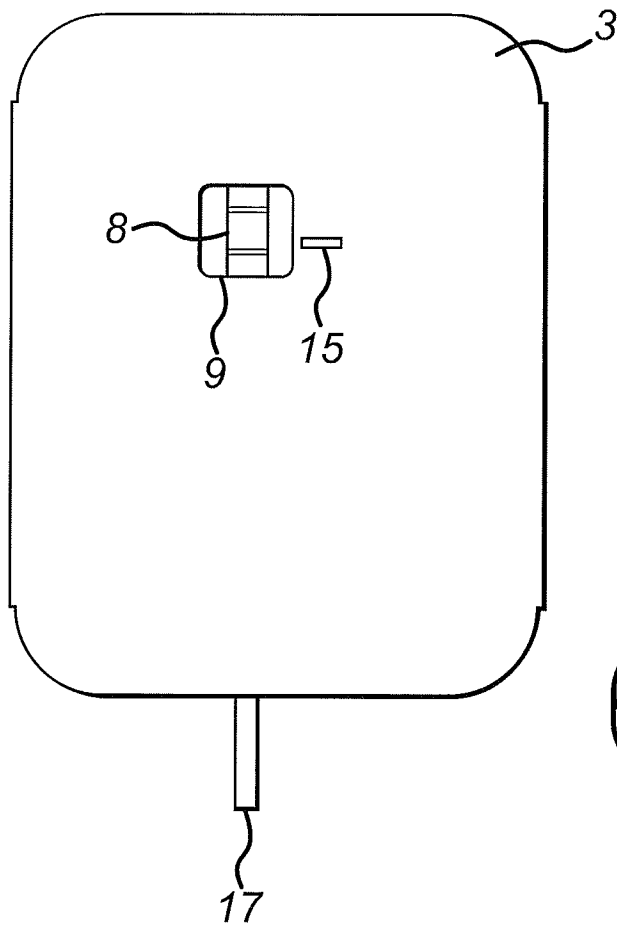


FIG. 11

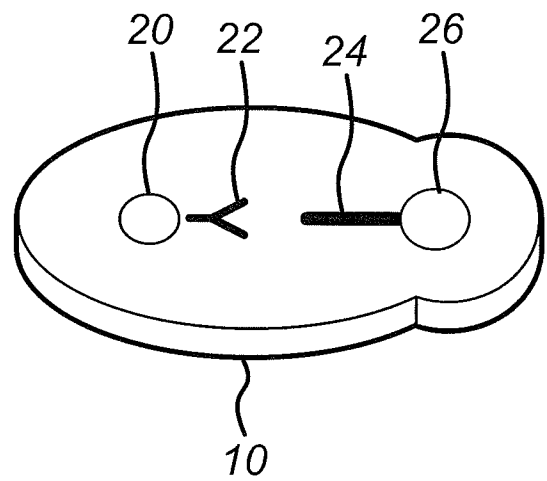


FIG. 15

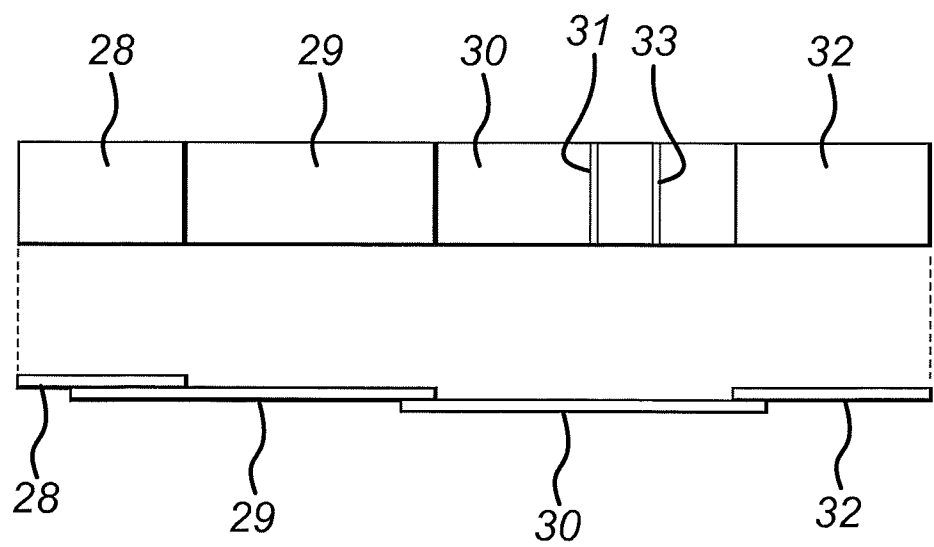
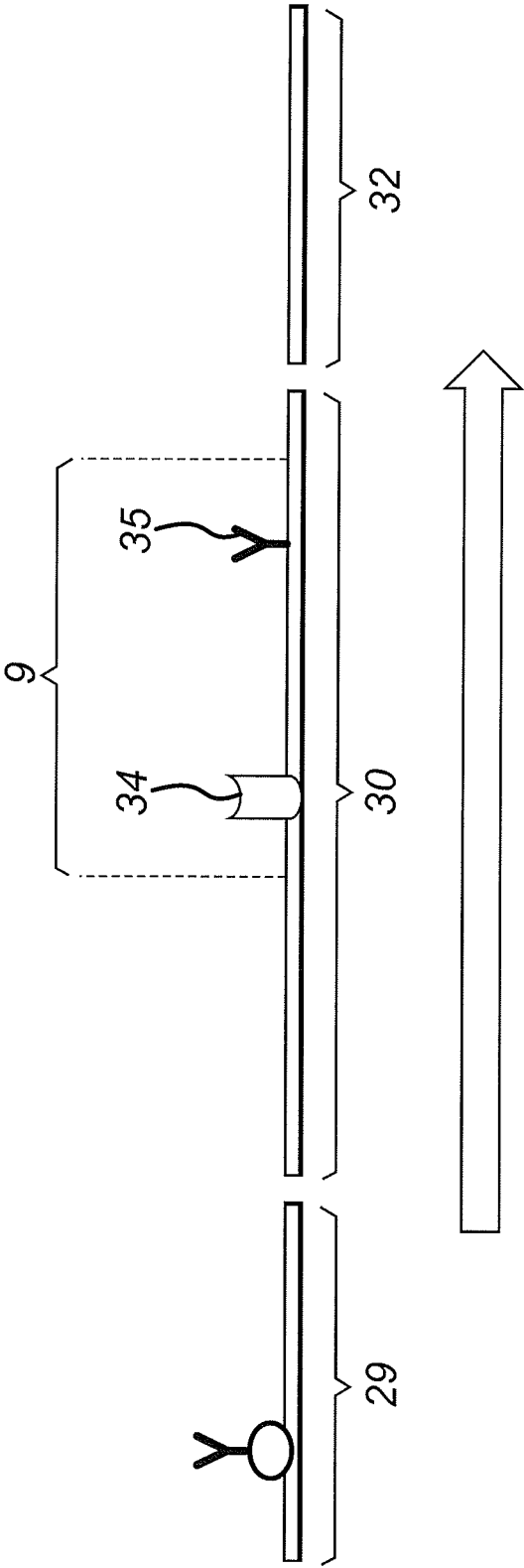


FIG. 12



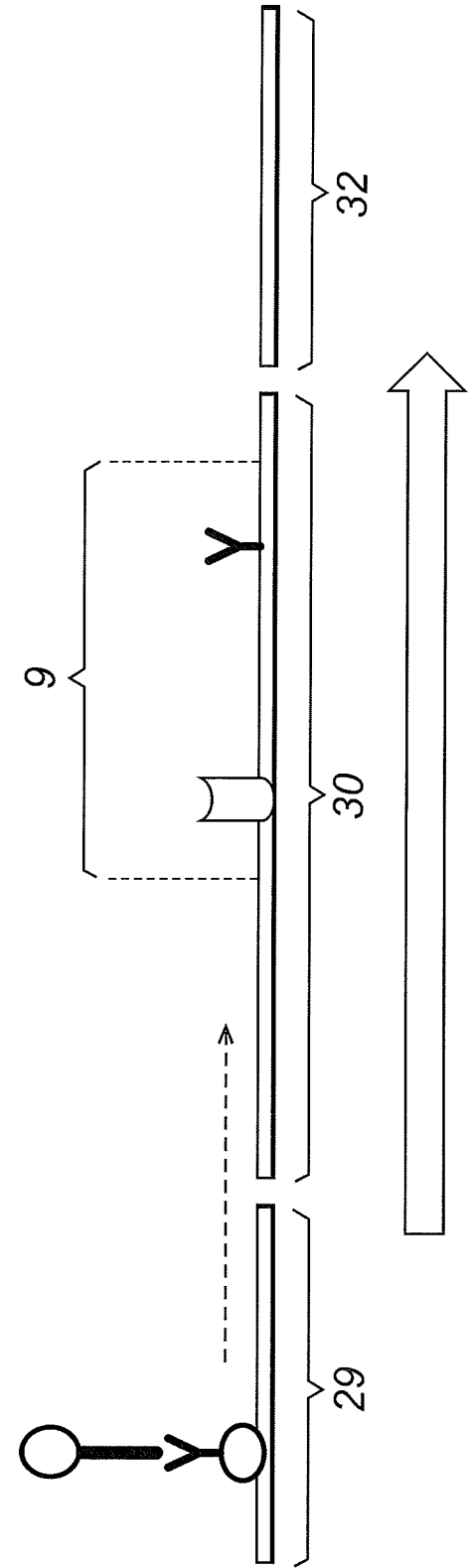


FIG. 13(a)

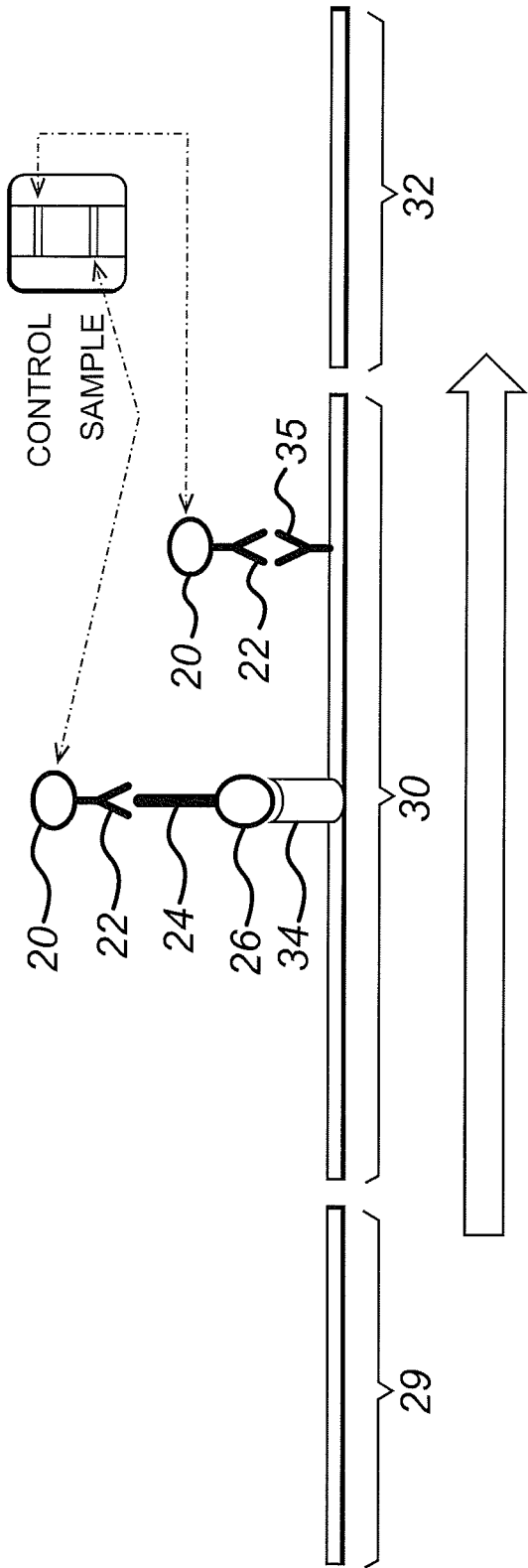


FIG. 13(b)

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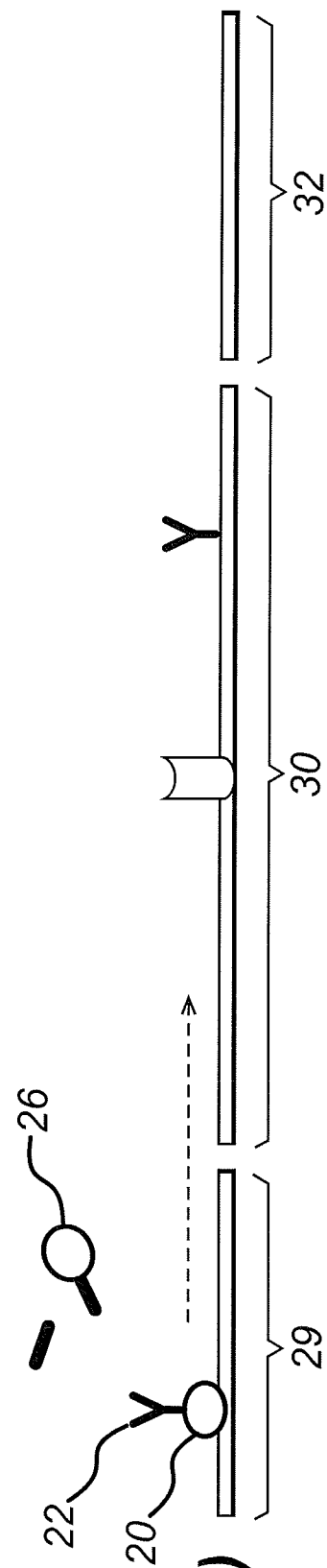


FIG. 14(a)

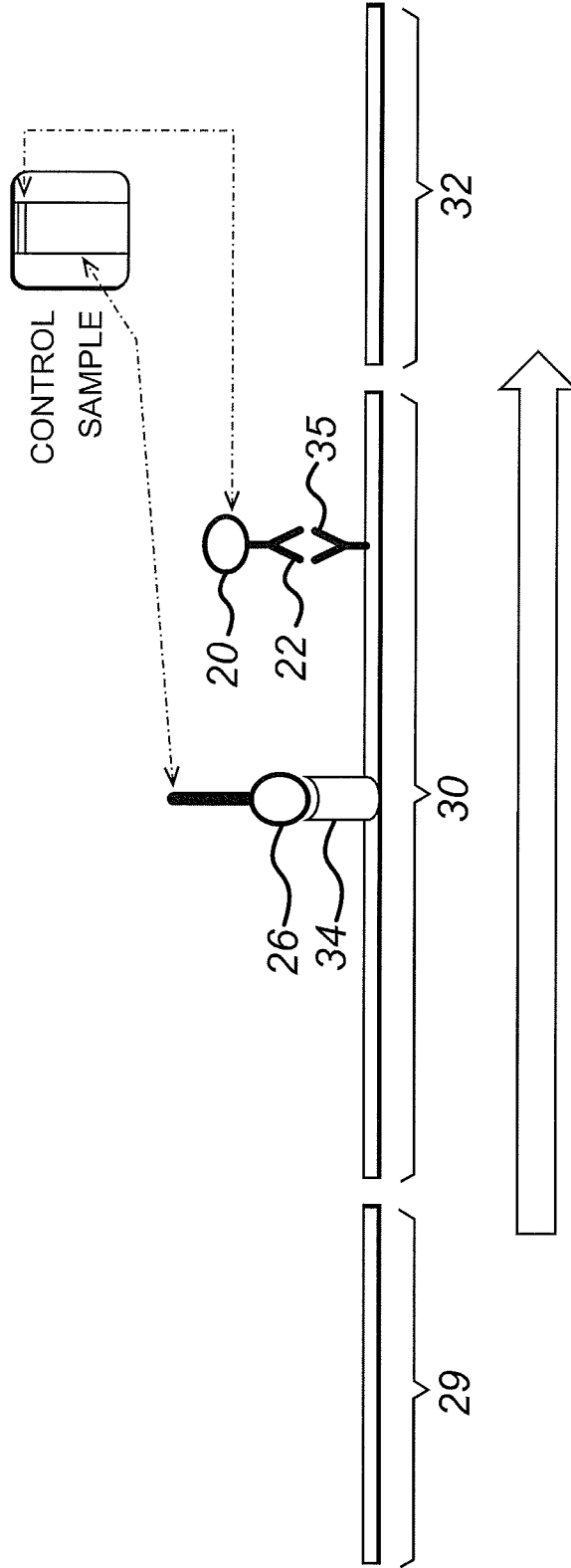


FIG. 14(b)