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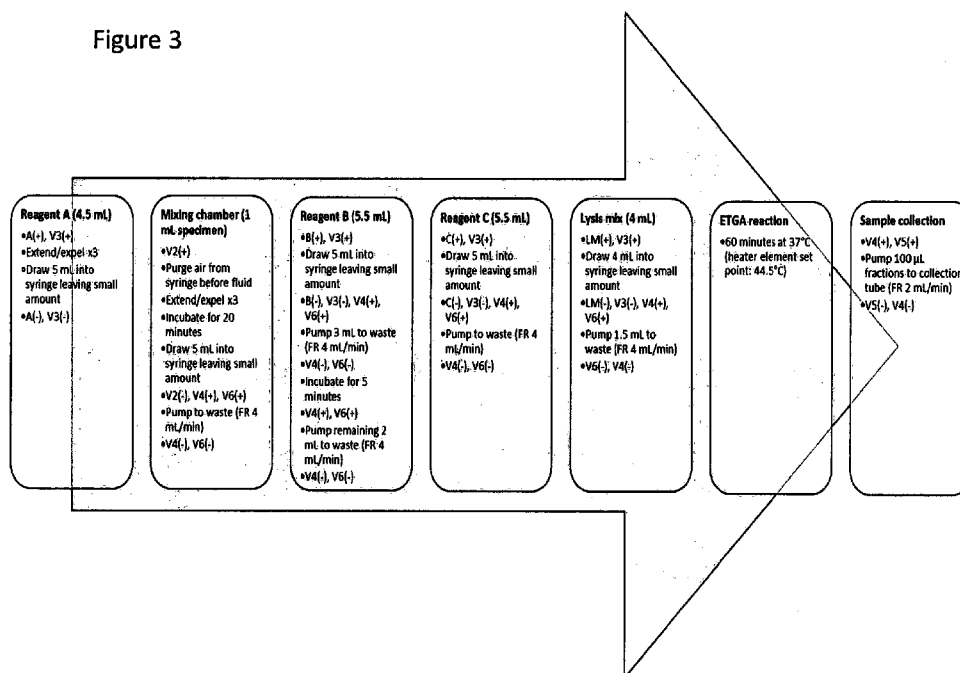
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(54) Title: MICROORGANISM DETECTION INVOLVING FILTRATION

Figure 3



(57) **Abstract:** A method of detecting the absence or presence of a microorganism is performed in a sample that may also contain non-microorganism cells. The method comprises selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample. The lysate is filtered through a first filter that prevents passage of insoluble cellular material through the filter. The filtrate is then filtered through a second filter that retains microorganisms within or upon the filter. The absence or presence of microorganisms retained within or upon the filter can then be detected. The first and second filter advantageously is provided in a single filter structure that may comprise asymmetric pores or may be a laminate structure. Larger pores on the inlet side prevent passage of insoluble material (arising from lysis of non-microorganism cells) but allow intact microorganisms to pass to the outlet side of the filter. The outlet side of the filter has smaller pores to thereby trap the microorganisms. Related kits and products



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MICROORGANISM DETECTION INVOLVING FILTRATION

FIELD OF THE INVENTION

The present invention relates generally to the field of detecting the absence or presence of microorganisms in a sample. The methods typically rely upon measuring microbial enzyme activity (if any) present in a sample where the sample also contains non-microorganism sources of enzyme activity. The invention relies upon effective isolation of the microorganism source of enzymatic activity. The methods of the invention therefore enable determination of the absence and presence of microbial pathogens in samples such as unpurified blood, blood culture and other body fluids. This invention also relates to devices, compositions of matter and kits comprising such reagents useful for carrying out the methods.

BACKGROUND TO THE INVENTION

Measuring the presence and levels of certain molecules which are associated with cell viability is important in a number of contexts. For example, measuring levels of ATP is useful in mammalian cells for growth analysis and toxicology purposes. Culture approaches can be used to detect small numbers of bacteria but such techniques require several days to complete, especially when attempting to detect small numbers of bacteria and also when detecting slower growing microorganisms.

Detection of adenylate kinase as an indicator of viability has also been proposed (Squirrell DJ, Murphy MJ, Leslie RL, Green JCD: A comparison of ATP and adenylate kinase as bacterial cell markers: correlation with agar plate counts). WO96/002665 describes a method for determining the presence and/or amount of microorganisms and/or their intracellular material present in a sample characterized in that the amount of adenylate kinase in the sample is estimated by mixing it with adenosine diphosphate (ADP), determining the amount of adenosine triphosphate (ATP) produced by the sample from this ADP, and relating the amount of ATP so produced to the presence/or amount of adenylate kinase and to microorganisms and/or their intracellular material, wherein the conversion of ADP to ATP is carried out in the presence of magnesium ions at a molar concentration sufficient to allow maximal conversion of ADP to ATP.

In WO2009/007719, NAD- dependent ligases are described as a useful indicator of the presence of a microorganism in a sample. Ligases are enzymes which catalyze ligation of

nucleic acid molecules. The ligation reaction requires either ATP or NAD⁺ as co-factor depending upon the ligase concerned.

WO2011/130584 describes a method for detection of viable microorganisms based on detection of DNA or RNA polymerases in which a sample is contacted with a nucleic acid substrate that acts as a substrate for microbial polymerase, incubated under conditions suitable for polymerase activity from intact microorganisms and any resulting nucleic acid product is determined using a nucleic acid amplification technique such as quantitative polymerase chain reaction. Such assays have been termed "ETGA assays", where ETGA stands for Enzymatic Template Generation and Amplification. A problem with ETGA assays for viable microorganisms in crude samples is the presence of contaminating polymerase activity outside the microorganisms arising from host (e.g. human) cells and dead microorganisms. The ETGA assay is unable to distinguish microorganism polymerase activity from that of the host or from dead microorganisms.

WO2010/119270 describes a method for removing DNA ligase activity outside intact microorganisms.

WO2011/070507 describes the selective lysis of animal cells using a non-ionic detergent and a buffer.

DESCRIPTION OF THE INVENTION

The present inventors have recognised that in samples taken from subjects suspected of carrying a microbial infection there are much greater levels of nucleated blood cells (leukocytes) than previously imagined even though the majority of samples are not in fact from infected subjects. This has led to the requirement for improved methods of separating potential microbes from blood cells, in particular leukocytes, in blood samples taken from patients screened for infection. Pre-filtering the lysate following lysis of non-microorganism cells and prior to capture of microorganisms for detection provides significant technical advantages as discussed herein. This is particularly the case when implementing automated, or semi-automated methods, in which sample handling is intended to be minimised.

Accordingly, in a first aspect, the invention provides a method of separating microorganisms from non-microorganism cells in a non-microorganism cell-containing sample, the method comprising:

- 5 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter.

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Such methods are particularly useful in the context of methods for detecting whether or not there is a microbial infection in the sample. Therefore, the invention also provides a method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising:

- 15 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter
- 20 d. detecting the absence or presence of microorganisms retained within or upon the filter.

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The detection of the absence or presence of microorganisms retained within or upon the filter can be performed according to any desired method. The method may involve detecting the simple absence or presence of the one or more microorganisms. It may involve quantification of the microorganisms, if present. It may also involve characterisation of the nature of the microorganism in some embodiments. Thus, detection of bacteria and/or fungi may be performed. Discrimination of gram positive versus gram negative bacteria may also be performed. Detection may occur on the filter or may occur following recovery of the retained microorganisms from the filter. Recovery may be of the intact microorganisms or of a lysate following lysis of the microorganisms (as discussed in further detail herein). Recovered microorganisms may be lysed prior to detection.

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The detection of the absence or presence of microorganisms retained within or upon the filter may comprise detecting an enzymatic activity or a nucleic acid molecule associated with the microorganism; detecting the microorganism directly by cytometry or microscopy; or detecting the microorganism following cell culture.

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Detection of nucleic acid molecules associated with microorganisms is known in the art and may be performed at the DNA or RNA level. It can be performed by any suitable method, such as amplification (e.g. PCR) or sequencing (in particular next generation sequencing). Such methods may take advantage of sequence divergence between microorganisms and non-microorganisms, such as human, DNA and RNA. Such methods may involve lysing the microorganisms on the filter in order to release the nucleic acid component. This may then be measured in the filter or, more preferably, in a separate reaction vessel after recovery from the filter.

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15 Direct detection of microorganisms is also known. This may involve cytometric analysis, for example by flow cytometry. It may involve use of microscopy, for example to visualise the microorganisms retained within or upon the filter.

Microorganism detection may also be performed following cell culture, in order to expand the number of microorganisms. Thus, the microorganisms initially captured upon or within the filter can be cultured for a set period of time, either upon or within the filter or elsewhere, prior to detection. Culture methods may permit direct detection of microorganisms in the original sample.

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25 However, in preferred embodiments, the detection of the absence or presence of microorganisms retained within or upon the filter may comprise detecting an enzymatic activity associated with the microorganism. Suitable enzymatic activities are typically nucleic acid modifying activities and are discussed in greater detail herein. Accordingly, in some embodiments, the methods of the invention comprise a step of detecting the absence or presence of microorganisms retained within or upon the filter (step d) which comprises steps of:

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- i. lysis of the microorganisms retained within the filter following step c
 - ii. incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms;
- and

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iii. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

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The invention therefore provides a method of detecting the absence or presence of a (viable or recently viable) microorganism in a sample that may also contain non-microorganism cells comprising:

- a. selective lysis of non-microorganism cells in the sample whilst retaining
10 intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter
- 15 d. lysis of the microorganisms retained within or upon the filter following step c
- e. incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
- f. specifically determining the absence or presence of a modified nucleic acid
20 molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

A key feature of the invention is selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample. This is important to
25 prevent enzymatic activity from non-microorganism cells, such as leukocytes, falsely indicating the presence of microorganisms in the sample. Such selective lysis can be achieved by any suitable means as discussed further herein. Any suitable reagent that lyses non-microorganisms, in particular mammalian cells, present in the sample but does not lyse microorganisms in the sample may be utilised. The reagent may include a
30 surfactant or detergent in some embodiments, such as a non-ionic detergent. Suitable examples include polyethylene glycol sorbitan monolaurate (Tween 20), for example at 5% w/v. The reagent may include a saponin, for example at 5% w/v. The reagent may include a metal halide salt, such as sodium chloride, for example at 8.5g/l. The reagent may include a mixture of all three components. The sample may be mixed with the reagent
35 under suitable conditions to ensure lysis of non-microorganism cells, in particular

mammalian cells, if present in the sample but no (or insignificant) lysis of microorganisms if present in the sample. The sample may be exposed to the reagent for a period of between around 5 and 30 minutes, such as 5, 10, 15, 20, 25 or 30 minutes. This step may be performed at any suitable temperature, for example between 15 and 30 degrees Celsius or at room temperature.

In some embodiments, according to all aspects of the invention, selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample comprises adding a combination of a detergent and one or more enzymes to the sample. Without wishing to be bound by any particular theory, the detergent selectively permeabilises non-microorganism cell membranes, whereas the microorganisms are protected by virtue of their cell wall. The enzymes are useful for breaking down released intracellular material and other cellular debris and may contribute to preventing carry over of released enzymatic activity. In some embodiments, the one or more enzymes comprise a proteinase and/or a nuclease. Suitable proteinases include proteinase K. Suitable nucleases include DNAses. In one embodiment, the reagent used to selectively lyse non-microorganism cells comprises a combination of triton X-100 and proteinase K. More specifically the lysis reagent may comprise 0.25% Triton X-100 and 4.8 µg/mL Proteinase K.

The inventors have discovered that use of filtration of the sample, in particular two distinct filtration steps, as part of the method may be preferable over other separation methods such as centrifugation, particularly as part of efforts to automate the methods. Thus, in some embodiments, the methods are semi- or fully-automated methods. In some embodiments, those methods are run in parallel fashion. Thus, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more assays may be run in parallel. High throughput methods may be performed. Such methods may be performed using the devices, compositions of matter and kits of the invention as described herein. The methods of the invention may comprise, following selective lysis of the non-microorganism cells in the sample, a step of filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter.

The first filter thus functions as a coarse filter to prevent large insoluble matter from passing, and thus impacting on the subsequent microorganism enzymatic activity detection methods. In particular, the first filter prevents blockage of the second filter. Even where

enzymes are included in the lysis procedure, there will typically be a significant amount of insoluble cellular material. As discussed herein, the inventors have discovered that blood samples even from subjects not carrying an infection typically contain relatively high levels of nucleated blood cells (i.e. leukocytes). This presents a practical problem when
5 attempting to perform the methods of the invention. "Insoluble cellular material" is thus defined herein as a by-product of cell lysis (i.e. cellular debris) that is not broken down by the lysis reagents utilised (to lyse non-microorganism cells in the sample). In some embodiments, the insoluble cellular material comprises, or is, precipitated nuclear material, such as chromatin. Suitable filter materials and pore sizes etc. to effectively remove such
10 material from the lysate are discussed in further detail herein.

The initial filtration thus generates a filtrate. This filtrate is then further filtered through a second filter. The second filter has the functional requirement of retaining any microorganisms contained in the filtrate within or upon the filter. This permits
15 microorganism lysis within or upon the filter, which the inventors have found to be particularly advantageous in the context of automation of their assays.

While the first and second filters may be physically separated from one another, permitting sequential filtration steps in which the first filtrate is recovered and then applied to the
20 second filter, this is not essential. Thus, by "first" and "second" filters is simply meant filtration materials with relevant properties permitting the functions specified for each filter. Those functions may be achieved in the context of a single overall filter, or single filtration body (which terms are considered interchangeable), containing two distinct structural and functional parts. Accordingly, in some embodiments of the invention, the two filtration steps
25 (steps b and c) are performed using a single filter, or single filtration body, comprising a pore size on the inlet side of the filter/filtration body that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter/filtration body that retains microorganisms within the filter. Thus, as the lysate is added to the inlet side of the filter/filtration body, the pores are dimensioned to prevent passage of insoluble cellular
30 material through the filter/filtration body. However, the remainder of the lysate passes through the filter/filtration body. The pores on the outlet side of the filter/filtration body are dimensioned such that any (intact) microorganisms in the lysate will not exit the filter/filtration body. Instead, they will remain trapped within the filter/filtration body, but separated from the insoluble cellular material, to permit the relevant further steps of the

assay to be performed. Thus, the filter/filtration body may be of a thickness sufficient to permit separation of the insoluble cellular material from the captured microorganisms.

In some embodiments according to all aspects of the invention, the filter/filtration body comprises an asymmetric pore structure. Thus, the filter/filtration body may comprise pores that (gradually) decrease in size between the inlet and outlet sides of the filter. In this respect, the first and second filters are functionally, but not necessarily physically, separable. In other embodiments, the first and second filters are formed through a laminate structure. Thus, in some embodiments, the two filtration steps (steps b and c) are performed using a filter/filtration body comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter/filtration body.

Thus, in some embodiments, a method of separating microorganisms from non-microorganism cells in a non-microorganism cell-containing sample, comprises:

- a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through
 - i. a single filter/filtration body comprising a pore size on the inlet side of the filter/filtration body that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter/filtration body that retains microorganisms within the filter/filtration body; or
 - ii. a filter/filtration body comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter/filtration body

Similarly, in some embodiments, a method of detecting the absence or presence of a (viable or recently viable) microorganism in a sample that may also contain non-microorganism cells comprises:

- a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through
 - i. a single filter/filtration body comprising a pore size on the inlet side of the filter/filtration body that prevents passage of insoluble cellular

- material and a pore size on the outlet side of the filter/filtration body that retains microorganisms within the filter/filtration body; or
- ii. a filter/filtration body comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter/filtration body
- c. lysis of the microorganisms retained within the filter/filtration body
- d. detecting the absence or presence of microorganisms retained within or upon the filter.

Any suitable detection method may be employed as discussed herein. In some embodiments, a method of detecting the absence or presence of a (viable or recently viable) microorganism in a sample that may also contain non-microorganism cells comprises:

- a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through
- i. a single filter/filtration body comprising a pore size on the inlet side of the filter/filtration body that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter/filtration body that retains microorganisms within the filter/filtration body; or
- ii. a filter/filtration body comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter/filtration body
- c. lysis of the microorganisms retained within the filter/filtration body
- d. incubating the lysate from step c with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
- e. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

Any suitable filter type may be employed. In some embodiments, the filter/filtration body is a membrane. Suitable membrane materials are discussed herein and include polysulfone

membranes, such as polyethersulfone membranes (e.g. available from Merck Millipore). Other membrane materials that may be utilised include polyvinylidene fluoride (PVDF) membranes (e.g. available from Merck Millipore) and glass fibre filters, optionally with a binder (e.g. available from Merck Millipore). Each may be provided in asymmetric form or
5 as a laminate structure as discussed herein. Each may incorporate pores of the specific sizes as described in further detail herein.

Pore sizes are typically presented in terms of (maximum) "diameter". However, the sizes herein generally refer to the maximum dimension of the pore to take account of the fact that
10 different filters may contain non-circular pores. This dimension may represent the intended average of all pores in the filter, based on the usual manufacturing tolerances and the use of non-uniform materials, as would be readily appreciated by one skilled in the art. Suitable pore sizes to prevent passage of insoluble cellular material may be, or may be on average, at least 1 μm . They may be, or may be on average, between 1 μm and 5 μm , such as
15 between 1 μm and 2 μm . Thus, in some embodiments step b comprises use of a filter with a (minimum) pore size (on the inlet side) of at least 1 μm . In certain embodiments, the (minimum) pore size (on the inlet side) is between 1 μm and 5 μm , such as between 1 μm and 2 μm .

20 Suitable pore sizes to achieve retention of microorganisms on or within the filter may be, or may be on average no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than 0.2 μm . Thus, in some embodiments, step c comprises use of a filter with a (maximum) pore size (on the outlet side) of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more
25 than 0.2 μm . A particularly suitable membrane type tested herein is an asymmetric polysulfone membrane with a pore size of around 0.22 μm (on the outlet side).

As already mentioned, it is important to inactivate any relevant enzymatic activity released when the non-microorganism cells are lysed. The inventors have devised methods in
30 which high pH conditions are utilised to ensure effective inactivation of the enzymatic activity. The microbial cells typically remain intact, at least during some of the treatment, and intracellular enzymatic activity is not significantly adversely affected by the high pH treatment. In addition, the inventors have previously shown that microbial enzymes are more resistant to the high pH treatment in any case. Thus, in some embodiments of the
35 invention, the second filter is exposed to high pH conditions. This also means that the

captured microorganisms (upon or within the second filter) are exposed to high pH conditions.

5 The duration of exposure to the high pH conditions is typically less than 20 minutes and may be not more than 10, 9, 8, 7, 6 or 5 minutes and may be around 5, 6, 7, 8, 9 or 10 minutes. In some embodiments the treatment is carried out for between around 2 and 15 minutes, such as around 5 minutes. By “around” is meant plus or minus 30 seconds.

10 Any suitable reagent may be added to the second filter (containing the captured microorganisms) in order to provide high pH conditions. In particular embodiments, the high pH conditions comprise contacting the sample with an alkali or a buffer. In particular embodiments, NaOH or Na₂CO₃ is used. In specific embodiments, the concentration of the NaOH or Na₂CO₃ is around 5mM or greater. The buffer may have a pKa value above 9. Examples of suitable buffers include borate, carbonate and pyrophosphate buffers.

15 The high pH conditions typically inhibit the activity of nucleic acid modifying enzymes including ATP-dependent ligase and polymerases from non-microorganism sources such as mammalian cells, but do not inhibit the activity of the microbial ligases or polymerases. This is primarily due to the differential lysis conditions employed in the methods to ensure
20 that only the non-microorganism enzymes are exposed to the high pH conditions. However, it may also be due to the greater resistance of microbial enzymes to these conditions. “High pH” is generally a pH of at least around 10, such as around 10, 11, 12, 13 or 14. “Low pH” is generally a pH of less than or equal to around 4, such as around 4, 3, 2, or 1. By “around” is meant 0.5 of a pH unit either side of the stated value. Altering the
25 pH of the sample may be achieved using any suitable means, as would be readily appreciated by one skilled in the art. Microbial enzymes such as polymerases and ligases may be resistant to extremes of pH, whereas corresponding mammalian enzymes may be inactivated under the same pH conditions. This assists with the selective detection of microbial enzymatic activity in a sample containing both mammalian cells and microbial
30 cells. In specific embodiments, the conditions that inhibit the activity of non-microorganism nucleic acid modifying activity, such as ATP-dependent ligase, from mammalian cells but which do not inhibit the activity of the microorganism source of nucleic acid modifying activity, such as microbial ligases, comprise treating the sample with sodium hydroxide (NaOH) or sodium carbonate (Na₂CO₃). Such agents can readily be used, as shown
35 herein, to increase the pH of the sample to high pH thus inactivating non-microorganism

enzymatic activity whilst leaving the microbial (fungal and bacterial) enzymes active.

Suitable concentrations and volumes of the appropriate agent can be applied by a skilled person. In certain embodiments, however, the NaOH is at least around 5mM NaOH. In some embodiments, the alkali concentration is no more than 10mM, such as 5, 6, 7, 8, 9 or 10mM.

In further embodiments, the pH is around 12 to inactivate mammalian nucleic acid modifying activity (such as polymerase and/or ATP-dependent ligase activity), but not microbial nucleic acid modifying activity (such as polymerase and/or ligase activity). In specific embodiments, pH conditions may be increased to at least around 11, or at least 11.2. This treatment may, after a certain period of time, result in lysis of microorganisms in the sample and thus lead to nucleic acid modifying activity (e.g. polymerase and/or ligase) release into the sample. Thus, in some embodiments, the lysis of microorganisms is achieved by high pH treatment. This permits detection of nucleic acid modifying activity (e.g. polymerases and/or ligases) in the sample, originating from the microorganism, without the need for a separate cell lysis step. Under these conditions, mammalian ligases (such as blood ATP-dependent ligases) are inactivated. However, typically the methods include a separate step for lysing microorganisms in the sample, as discussed in greater detail herein.

In some embodiments, the treatment under high pH conditions is stopped by adding a reagent to lower the pH. This is done before the microorganisms are lysed. Thus, according to further embodiments, the pH is subsequently reduced before step d is performed. Suitable reagents include a buffer and/or an acid. Thus, the pH may be reduced by adding a neutralisation buffer. In specific embodiments, the buffer comprises a Tris-HCl buffer (e.g. pH 7.2 or 8). Other suitable agents for lowering the pH include acids such as hydrochloric acid (HCl) and sulphuric acid (H₂SO₄). These (and other) acids may be incorporated into a buffer as would be readily appreciated by one skilled in the art. One specific reagent useful for treating the sample after the pH has been elevated comprises a combination of Ammonium sulphate, Magnesium sulphate heptahydrate, Potassium chloride and Tris-HCl. More specifically, the reagent may comprise 10 mM Ammonium sulphate, 2 mM Magnesium sulphate heptahydrate, 10 mM Potassium chloride and 20 mM Tris-HCl [pH 8.0].

The methods of the invention may require steps of lysis of the microorganisms retained within or upon the filter and incubating the resulting lysate with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms. In some embodiments these steps of the method overlap; lysis of the microorganisms may be performed using a lysis reagent containing the substrate nucleic acid molecule. In such 5 embodiments, a volume of lysis reagent is typically applied that will saturate the filter. In additional or alternative embodiments the volume applied to the filter is no more than 1.1, 1.2, 1.3, 1.4 or 1.5 times the capacity of the filter. This helps to prevent enzymes released from the lysed microorganisms being washed out of the filter (to a significant degree). The 10 additional volume (i.e. moderately greater than capacity) may be beneficial to ensure previous solution contained within the filter is forced out. The additional volume may thus ensure saturation with a substantially undiluted lysis reagent. In additional or alternative embodiments, any active process (e.g. pumping) for applying the lysis reagent to the filter is stopped or otherwise controlled so as to prevent enzymes released from the lysed 15 microorganisms being forced out of the filter (to a significant degree). These steps are advantageous because it is generally preferred that the incubation with the substrate nucleic acid molecule is performed in the filter. For example, in a reaction volume of 200 µl, the methods may involve pumping 250 µl of lysis reagent onto the filter.

20 The methods of the invention may thus incorporate the following steps:

- a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- 25 c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter
- d. lysis of the microorganisms retained within or upon the filter following step c using a lysis reagent, wherein the lysis reagent includes a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity
- 30 e. incubating the lysate from step d with the substrate nucleic acid molecule; and
- f. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of 35 the microorganism.

Lysis of microorganisms retained within or upon the filter permits detection of nucleic acid molecules or enzymes within the microorganisms, such nucleic acid modifying enzymes. Lysis may be achieved by addition of a lysis mixture. The lysis mixture is generally useful
5 in the methods of the invention. The lysis mixture may include a specific mixture of components to ensure efficient lysis of microorganisms without adversely affecting nucleic acid molecules and/or enzyme activity, such as nucleic acid modifying activity, within the cells. The components may be selected from carrier/serum proteins such as BSA, surfactants/detergents, metal halide salts, buffers, chelators etc. In its basic form, the lysis
10 mixture of the invention may include the following components:

1. A surfactant/detergent
2. Serum protein such as albumin (e.g. BSA)
3. Buffer
4. Nucleotides, such as dNTPs
- 15 5. Nucleic acid molecule (acting as a substrate in the assays of the invention).

A suitable lysis mixture is set forth below:

L1: 252 mL in 360 mL LM

- 20 1.46% (w/v) BSA
0.15% Triton X100
0.15% Tween 20

L2: 36 mL in 360 mL LM

- 25 100 mM Ammonium sulphate
20 mM Magnesium sulphate heptahydrate
100 mM Potassium chloride
200 mM Tris-HCl [pH 8.0]

L3: 36 mL in 360 mL LM

- 30 0.1 μ M PTO-AS oligo
0.1 μ M PTO-S1 oligo
20 mM Tris-HCl [pH 8.5]
10 mM Potassium chloride
35 10 μ M EDTA

10 mM dNTPs: 3.6 mL in 360 mL LM

PTO-IPC stock: ~180 μ L in 360 mL LM

5

H₂O: ~32.4 mL in 360 mL LM

By "PTO-AS oligo" is meant an antisense oligonucleotide comprising phosphorothioate nucleotides. By "PTO-S1 oligo" is meant a sense oligonucleotide comprising
10 phosphorothioate nucleotides. The two oligonucleotides hybridise to one another to form the substrate nucleic acid molecule.

By "PTO-IPC" is meant an IPC molecule comprising phosphorothioate nucleotides.

15 Suitable substrate and IPC molecules are discussed in further detail herein.

Exemplary amounts and concentrations of each component are listed but may be modified as would be readily appreciated by one skilled in the art.

20 Lysis may also require disruption of the cells. For example, the cells may be disrupted using the lysis mixture in combination with physical and/or enzymatic means. Typically, however, the methods in which the cells are lysed in or on the filter avoid use of physical disruption (other than any active process, such as pumping, which applies the lysis mixture to the filter). In some embodiments, physical disruption employs a disruptor. The disruptor
25 may incorporate beads such as glass beads to lyse the cells. Suitable apparatus are commercially available and include the Disruptor Genie manufactured by Scientific Industries, Inc. Sonication may be utilised, for example applying a(n ultra) sonic horn. Enzymatic disruption may require use of one or more agents selected from lysostaphin, lysozyme and/or lyticase in some embodiments.

30

Once the microorganisms, if present in the sample, are lysed, the released nucleic acid and/or enzymes may be detected to indicate whether microorganisms are present in the sample. In some embodiments, the lysate is incubated with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity (of the microorganisms). The absence
35 or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid

modifying enzyme on the substrate nucleic acid molecule is then determined to indicate the absence or presence of the microorganism. The nucleic acid substrate molecule is designed according to the nucleic acid modifying activity that is to be detected. One skilled in the art is well able to design suitable substrate nucleic acid molecules. Although the
5 initial sample contains non-microorganism sources of nucleic acid modifying activity, the methods of the invention prevent this contaminating activity acting on the substrate nucleic acid molecules.

According to the methods of the invention typical nucleic acid modifying activity that may be
10 detected comprises polymerase and/or ligase activity. In certain embodiments, nucleic acid modifying enzyme comprises DNA or RNA polymerase. In some embodiments, the DNA polymerase comprises or is DNA polymerase I. In some embodiments, the nucleic acid modifying enzyme comprises a ligase. In certain embodiments, the nucleic acid modifying enzyme comprises or is an NAD-dependent or ATP-dependent ligase. NAD-
15 dependent ligases are only found in (eu)bacteria and thus, detecting such activity may provide an additional level of specificity. This is discussed further in WO2009/007719 and WO2010/119270 (the pertinent disclosures of which are hereby incorporated). Other nucleic acid modifying activities relevant to viability may alternatively be measured such as phosphatase, kinase and/or nuclease activity.

20 In some embodiments, the action of the nucleic acid modifying activity on the substrate nucleic acid molecule produces an extended nucleic acid molecule. This may be by strand extension (polymerase activity) and/or by ligation of two nucleic acid molecules (ligase activity). In some embodiments, a substrate that can be acted upon by either polymerase
25 or ligase is utilised since either activity is indicative of the presence of a microorganism in the sample. In some embodiments, the relevant activity can be distinguished in terms of the novel nucleic acid molecule that is produced.

Suitable substrate molecules are described in WO2011/130584, WO2010/119270 and
30 WO2009/007719 (the pertinent disclosures of which are hereby incorporated). In the case of phosphatase activity, suitable nucleic acid molecules are disclosed in WO2006/123154, which disclosure is hereby incorporated by reference.

In specific embodiments, the (substrate) nucleic acid molecule used in the methods of the
35 invention is at least partially double stranded and comprises uracil residues in the

complementary strand and the step of specifically determining the absence or presence of the modified nucleic acid molecule comprises adding Uracil DNA Glycosylase (UDG) to the sample in order to degrade the uracil residues in the complementary strand.

- 5 In certain embodiments, the first strand of the partially double stranded (substrate) nucleic acid molecule comprises (or consists of) synthetic nucleotides (e.g. phosphorothioate nucleotides) and the second (complementary) strand comprises (or consists of) uracil residues and, optionally, synthetic nucleotides (e.g. phosphorothioate nucleotides). Preferably, the double stranded region encompasses the 3' end regions of the first and
- 10 second (complementary) strands. Preferably, the double stranded region is at least 5, at least 10, at least 15, at least 20 or at least 25 nucleotides; optionally, the double stranded region is no more than 50 nucleotides. The first strand may be extended during an incubation step, as described herein, using unprotected (or standard) dNTPs by the polymerase activity of a microorganism in the sample to form an extended first strand that
- 15 comprises unprotected (or standard) nucleotides. This step relies upon using the second strand as template (upstream of the region of complementarity between the first and second strands). Following the incubation step, the second (complementary) strand may be degraded by adding Uracil DNA Glycosylase (UDG) to the sample leaving the extended first strand as a single stranded molecule comprising synthetic nucleotides and unprotected
- 20 nucleotides. Following degradation of the second strand, the extended first strand of the (substrate) nucleic acid molecule may be detected in an amplification step. The inventors have found that the use of a partially double stranded (substrate) nucleic acid molecule as described above improves the detection of a microorganism in the sample.
- 25 In some embodiments, the substrate nucleic acid molecule is pre-modified so as to protect it from nuclease activity i.e. the nucleic acid molecule is modified so as to protect it from nuclease activity before it is added to the assay. The inventors have determined that protection of the substrate nucleic acid molecule from nuclease activity is advantageous in the context of the assays of the invention. More specifically, incorporation of protected
- 30 nucleic acid molecules into the methods of the invention improves sensitivity of detection. Any suitable means may be employed in order to protect the nucleic acid molecule from nuclease activity. Non-limiting examples include incorporation of methylation into the nucleic acid molecule, end modification such as protection of the 3' and/or 5' ends and incorporation of synthetic nucleotides. In specific embodiments, the synthetic nucleotides
- 35 comprise phosphorothioate nucleotides and/or locked nucleic acid nucleotides. Preferably,

the synthetic nucleotides are phosphorothioate nucleotides. In certain embodiments, the synthetic nucleotides replace at least one up to all of the nucleotides in the nucleic acid molecule.

- 5 The (substrate) nucleic acid molecules may include any natural nucleic acid and natural or synthetic analogues that are capable of being acted upon by nucleic acid modifying activity in order to generate a (novel detectable) nucleic acid molecule. The substrate may be extended and/or ligated in specific embodiments. Combinations of nucleic acid substrate molecules may be employed to permit detection of polymerase and ligase activity in some
10 embodiments.

The nucleic acid substrate may be present in excess, and in particular in large molar excess, over the nucleic acid modifying activity (provided by the microorganisms) in the sample. Because a novel extended or ligated nucleic acid molecule is detected, only the
15 presence of this molecule in the sample is essential for the detection methods to work effectively. Thus, it is not detrimental to the methods of the invention if other nucleic acid molecules are present in the sample such as from the microorganisms to be detected or from mammalian or other sources which may be found in the sample to be tested for example.

20

The inventors have previously investigated the use of an internal positive control (IPC) molecule in the context of their methods. Thus, according to all aspects, the invention may rely upon inclusion of an IPC molecule. In some embodiments, the IPC is included with the substrate nucleic acid molecule so that the IPC is exposed to identical conditions. In some
25 embodiments, the IPC molecule is pre-modified so as to protect it from nuclease activity i.e. the nucleic acid molecule is modified so as to protect it from nuclease activity before it is added to the assay. The inventors have determined that protection of the IPC molecule from nuclease activity is advantageous in the context of the assays of the invention. Any suitable means may be employed in order to protect the nucleic acid molecule from
30 nuclease activity. Non-limiting examples include incorporation of methylation into the nucleic acid molecule, end modification such as protection of the 3' and/or 5' ends and incorporation of synthetic nucleotides. In specific embodiments, the synthetic nucleotides comprise phosphorothioate nucleotides and/or locked nucleic acid nucleotides. Preferably, the synthetic nucleotides are phosphorothioate nucleotides. In certain embodiments, the
35 synthetic nucleotides replace at least one up to all of the nucleotides in the IPC molecule.

Preferably, the substrate and IPC molecules are modified in the same manner as it is advantageous for them to behave similarly in the assays of the invention.

5 In some embodiments, the internal positive control (IPC) nucleic acid molecule comprises identical primer binding sites to the substrate nucleic acid molecule such that there is competition for primer binding in a nucleic acid amplification reaction containing both the nucleic acid molecule and the IPC.

10 The step of incubating the lysate resulting from lysis of the microorganisms (from step d) with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms typically occurs on or in the filter. Thus, the lysate is retained within the filter and the nucleic acid molecule is added to the filter. In such embodiments, a volume of liquid containing the nucleic acid molecule (which may be the lysis reagent in some embodiments) is typically applied that will saturate the filter. In additional or
15 alternative embodiments the volume applied to the filter is no more than 1.1, 1.2, 1.3, 1.4 or 1.5 times the capacity of the filter. This helps to prevent enzymes released from the lysed microorganisms being washed out of the filter (to a significant degree). In additional or alternative embodiments, any active process (e.g. pumping) for applying the liquid containing the nucleic acid molecule to the filter is stopped or otherwise controlled so as to
20 prevent enzymes released from the lysed microorganisms being washed out of the filter (to a significant degree). These steps are advantageous because it is generally preferred that the incubation with the substrate nucleic acid molecule is performed in the filter. However, it is also possible that the incubation step can occur outside of the filter. This may involve capture of microorganism nucleic acid modifying activity released from the filter and adding
25 the nucleic acid molecule to the captured lysate. It may involve recovery of microorganisms from the filter before lysis takes place. In such embodiments, the nucleic acid molecule may be added as part of, or subsequent to, the lysis step.

The methods of the invention may incorporate steps of:

- 30 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- c. filtering the filtrate from step b through a second filter that retains
35 microorganisms within or upon the filter

- d. lysis of the microorganisms retained within or upon the filter following step c
- e. incubating the lysate from step d, contained within or upon the filter, with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
- 5 f. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

10 The step of determining the absence or presence of the modified nucleic acid molecule may potentially be performed on or in the filter where the incubation with the substrate nucleic acid molecule has taken place on or in the filter. However, the step of determining the absence or presence of the modified nucleic acid molecule is typically performed after the incubated sample has been recovered from the filter. It thus typically takes place in a
15 reaction vessel that is separate from the filter. The incubated sample may be pumped out of the filter into the reaction vessel. The determination step then takes place in the reaction vessel.

The methods of the invention may entail steps of:

- 20 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
- c. filtering the filtrate from step b through a second filter that retains
25 microorganisms within or upon the filter
- d. lysis of the microorganisms retained within or upon the filter following step c
- e. incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms;
- f. recovering the incubated lysate from the filter; and
- 30 g. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

In all methods of the invention specifically determining the absence or presence of the modified nucleic acid molecule may comprise, consist essentially of or consist of a nucleic acid amplification step. This serves to make the methods of the invention maximally sensitive. Such amplification techniques are well known in the art, and include methods
5 such as PCR, NASBA (Compton, 1991), 3SR (Fahy et al., 1991), Rolling circle replication, Transcription Mediated Amplification (TMA), strand displacement amplification (SDA) Clinical Chemistry 45: 777-784, 1999, the DNA oligomer self-assembly processes described in US6261846 (incorporated herein by reference), ligase chain reaction (LCR)
10 (Barringer et al., 1990), selective amplification of target polynucleotide sequences (US 6410276), arbitrarily primed PCR (WO 90/06995), consensus sequence primed PCR (US 4437975), invader technology, strand displacement technology and nick displacement amplification (WO 2004/067726). The list above is not intended to be exhaustive. Any nucleic acid amplification technique may be used provided the appropriate nucleic acid product is specifically amplified.

15 Similarly, sequencing based methodologies may be employed in some embodiments to include any of the range of next generation sequencing platforms, such as sequencing by synthesis of clonally amplified sequences (Illumina), pyrosequencing, 454 sequencing (Roche), nanopore sequencing (e.g. Oxford Nanopore), ion torrent (ThermoFisher) and
20 single molecule real-time (SMRT) sequencing (Pacific Biosystems). The fact that a novel nucleic acid molecule is generated means that a sequencing approach can confirm the presence or otherwise of the modified nucleic acid molecule and also provide quantification of that molecule.

25 Amplification is achieved with the use of amplification primers specific for the sequence of the modified nucleic acid molecule which is to be detected. In order to provide specificity for the nucleic acid molecules primer binding sites corresponding to a suitable region of the sequence may be selected. The skilled reader will appreciate that the nucleic acid molecules may also include sequences other than primer binding sites which are required
30 for detection of the novel nucleic acid molecule produced by the modifying activity in the sample, for example RNA Polymerase binding sites or promoter sequences may be required for isothermal amplification technologies, such as NASBA, 3SR and TMA.

One or more primer binding sites may bridge the ligation/extension boundary of the
35 substrate nucleic acid molecule such that an amplification product is only generated if

ligation/extension has occurred, for example. Alternatively, primers may bind either side of the ligation/extension boundary and direct amplification across the boundary such that an amplification product is only generated (exponentially) if the ligated/extended nucleic acid molecule is formed. Primers and the substrate nucleic acid molecule(s) may be designed to
5 avoid non-specific amplification (e.g. of genomic DNA in the sample).

Primers may incorporate synthetic nucleotide analogues as appropriate or may be RNA or PNA based for example, or mixtures thereof. The primers may be labelled, such as with fluorescent labels and/or FRET pairs, depending upon the mode of detection employed.
10

Probes may be utilised, again which may be labelled, as desired. The detection method may require use of nucleotide probes in addition to primers, or as an alternative to primers. For example, a branched DNA assay, which does not require use of primers, may be employed in some embodiments.
15

In certain aspects, the methods of the invention are carried out using nucleic acid amplification techniques in order to detect the modified nucleic acid molecule produced as a direct result of the action of nucleic acid-modifying activity on the substrate nucleic acid molecule which indicates the presence of a micro-organism in the sample. In certain
20 embodiments the technique used is selected from PCR, NASBA, 3SR, TMA, SDA and DNA oligomer self-assembly.

Detection of the amplification products may be by routine methods, such as, for example, gel electrophoresis but in some embodiments is carried out using real-time or end-point
25 detection methods.

A number of techniques for real-time or end-point detection of the products of an amplification reaction are known in the art. These include use of intercalating fluorescent dyes such as SYBR Green I (Sambrook and Russell, Molecular Cloning - A Laboratory
30 Manual, Third edition), which allows the yield of amplified DNA to be estimated based upon the amount of fluorescence produced. Many of the real-time detection methods produce a fluorescent read-out that may be continuously monitored; specific examples including molecular beacons and fluorescent resonance energy transfer probes. Real-time and end-point techniques are advantageous because they keep the reaction in a "single tube". This
35 means there is no need for downstream analysis in order to obtain results, leading to more

rapidly obtained results. Furthermore keeping the reaction in a "single tube" environment reduces the risk of cross contamination and allows a quantitative output from the methods of the invention. This may be particularly important in the context of the present invention where health and safety concerns may be of paramount importance (such as in detecting
5 potential microbial infection in a patient samples for example).

Real-time and end-point quantitation of PCR reactions may be accomplished using the TaqMan® system (Applied Biosystems), see Holland et al; Detection of specific
10 polymerase chain reaction product by utilising the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase; Proc. Natl. Acad. Sci. USA 88, 7276-7280 (1991), Gelmini et al. Quantitative polymerase chain reaction-based homogeneous assay with flurogenic probes to measure C-Erb-2 oncogene amplification. Clin. Chem. 43, 752-758 (1997) and Livak et al. Towards fully automated genome wide polymorphism screening. Nat. Genet. 9, 341-342 (19995) (incorporated herein by reference). This type of probe may be generically
15 referred to as a hydrolytic probe. Suitable hydrolytic/Taqman probes for use in real time or end point detection are also provided. The probe may be suitably labelled, for example using the labels detailed below.

In the Molecular Beacon system, see Tyagi & Kramer. Molecular beacons - probes that
20 fluoresce upon hybridization. Nat. Biotechnol. 14, 303-308 (1996) and Tyagi et al. Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. 16, 49-53 (1998) (incorporated herein by reference), the beacons are hairpin-shaped probes with an internally quenched fluorophore whose fluorescence is restored when bound to its target. These probes may be referred to as hairpin probes.

25 A further real-time fluorescence based system which may be incorporated in the methods of the invention is the Scorpion system, see Detection of PCR products using self-probing amplicons and fluorescence by Whitcombe et al. Nature Biotechnology 17, 804 - 807 (01 Aug 1999). Additional real-time or end-point detection techniques which are well known to
30 those skilled in the art and which are commercially available include Lightcycler® technology, Amplifluor® primer technology, DzyNA primers (Todd et al., Clinical Chemistry 46:5, 625-630 (2000)), or the Plexor™ qPCR and qRT-PCR Systems.

Thus, in further aspects of the invention the products of nucleic acid amplification are
35 detected using real-time or end point techniques. In specific embodiments of the invention

the real-time technique consists of using any one of hydrolytic probes (the Taqman® system), FRET probes (Lightcycler® system), hairpin primers (Amplifluor® system), hairpin probes (the Molecular beacons system), hairpin probes incorporated into a primer (the Scorpion® probe system), primers incorporating the complementary sequence of a DNAzyme and a cleavable fluorescent DNAzyme substrate (DzYNA), Plexor qPCR and oligonucleotide blocking systems.

Amplification products may be quantified to give an approximation of the microbial nucleic acid modifying activity in the sample and thus the level of microorganisms in the sample. Thus, “absence or presence” is intended to encompass quantification of the levels of microorganisms in the sample.

The inventors have further discovered that the optimal temperature for measuring nucleic acid modifying activity of the microorganisms may not be the same as the optimal temperature for lysis of microorganisms. Thus, in some embodiments, lysis of the microorganisms retained within or upon the filter following filtration through the second filter is performed at a lower temperature than the step of incubating the lysate with a nucleic acid molecule that acts as a substrate for nucleic acid modifying activity of the microorganisms. As already discussed, in some embodiments of the invention, the substrate nucleic acid molecule is included in the lysis reagent used to lyse the microorganisms. Such embodiments are consistent with the differing temperature preferences. Thus, even though the substrate nucleic acid molecule is included may be included in the lysis reagent, the initial lower temperature does not adversely affect the subsequent incubation at higher temperature, at which the substrate is modified by the nucleic acid modifying activity released from the microorganisms. Accordingly, in some embodiments the method involves a step of lysis of the microorganisms retained within or upon the filter following filtration through the second filter in which the lysis reagent contains a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms. This step is performed at a lower temperature than the subsequent step of incubating the lysate with the substrate nucleic acid molecule to enable the activity of the enzymes released from the microorganisms. Thus, the substrate is exposed to the initial lower temperature, followed by a higher temperature under which enzyme activity is enhanced.

In some embodiments, the step of incubating the lysate with a nucleic acid molecule that acts as a substrate for nucleic acid modifying activity of the microorganisms is performed at a temperature of at least around 30°C. The temperature may optionally be between around 30°C and 40°C or between around 32°C and 37°C, such as around 37°C.

5

In additional or alternative embodiments, the step of lysis of the microorganisms retained within or upon the filter following filtration through the second filter is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C. In

10 some embodiments, all steps prior to incubating the lysate with a nucleic acid molecule that acts as a substrate for nucleic acid modifying activity of the microorganisms are performed at a temperature of no more than around 30°C. The temperature may optionally be between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.

15

In one aspect of the invention, there is provided a method of detecting the absence or presence of a (viable or recently viable) microorganism in a sample that may also contain non-microorganism cells comprising:

- 20 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
 - b. separation of the intact microorganisms from the lysed cell material
 - c. lysis of the microorganisms
 - d. incubating the lysate from step c with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
 - 25 e. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism;
- characterised in that lysis of the microorganisms (step c) is performed at a
- 30 temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.

Such a method may incorporate any one or more up to all of the embodiments described in

35 relation to the various aspects of the invention.

In some embodiments, the method is further characterised in that the step of incubating the lysate with a substrate nucleic acid molecule (step d) is performed at a temperature of at least around 30°C, optionally between around 30°C and 40°C or between around 32°C and 37°C, such as around 37°C.

In additional or alternative embodiments, each of steps prior to incubating the lysate with a substrate nucleic acid molecule (i.e. steps a to c) is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.

The invention also relates to a method of detecting the absence or presence of a microorganism infection in a subject comprising performing a method of the invention on a sample from the subject. The detection of the modified nucleic acid molecule indicates whether there is an infection or not.

A "sample" in the context of the present invention is one which contains non-microorganism cells and in which it is desirable to test for the presence of a microorganism, such as a fungus (e.g. a yeast) or a bacterium, expressing nucleic acid modifying activity. Thus the sample may comprise, consist essentially of or consist of a clinical sample, such as a blood sample (to include whole blood, plasma, serum and blood containing samples, such as a blood culture). The methods of the invention are particularly applicable to the rapid determination of negative (and positive) blood cultures. Thus, the sample may comprise a blood culture sample from a patient suspected of suffering from, or being screened for, a bloodstream infection. The sample may be any suitable volume such as 1 to 10ml, preferably a 1ml blood culture sample.

The sample being used will depend on various factors, such as availability, convenience and the condition that is being tested for. Typical samples which may be used, but which are not intended to limit the invention, include whole blood, serum, plasma, platelet and urine samples etc. taken from a patient, most preferably a human patient. The patient may be suspected of suffering from, or being screened for, a bloodstream infection. The patient may be a hospitalised patient. The sample may be taken from a subject comprising more than 5, 10 or 15 million white blood cells (WBC) per ml of blood. The methods of the invention represent *in vitro* tests. They are carried out on a sample removed from a

subject. However, in less preferred embodiments, the methods may additionally include the step of obtaining the sample from a subject. Methods of obtaining a suitable sample from a subject are well known in the art. Typically, however, the method may be carried out beginning with a sample that has already been isolated from the patient in a separate
5 procedure. The methods will most preferably be carried out on a sample from a human, but the methods of the invention may have utility for many animals.

The methods of the invention may be used to complement any already available diagnostic techniques, potentially as a method of confirming an initial diagnosis. Alternatively, the
10 methods may be used as a preliminary diagnosis method in their own right, since the methods provide a quick and convenient means of diagnosis. Furthermore, due to their inherent sensitivity, the methods of the invention require only a minimal sample, thus preventing unnecessary invasive surgery. Also, a large but non-concentrated sample may also be tested effectively according to the methods of the invention.

15

In specific embodiments according to all aspects of the invention, the microorganism that may be detected in the sample is a pathogenic microorganism, such as a pathogenic bacterium or fungus/yeast. The bacterium may be any bacterium which is capable of causing infection or disease in a subject, preferably a human subject. In one embodiment,
20 the bacteria comprises or consists essentially of or consists of any one or more of *Staphylococcus* species, in particular *Staphylococcus aureus* and preferably methicillin resistant strains, *Enterococcus* species, *Streptococcus* species, *Mycobacterium* species, in particular *Mycobacterium tuberculosis*, *Vibrio* species, in particular *Vibrio cholerae*, *Salmonella* and/or *Escherichia coli* etc. The bacteria may comprise, consist essentially of
25 or consist of *Clostridium* species and in particular *C. difficile* in certain embodiments. *C. difficile* is the major cause of antibiotic-associated diarrhoea and colitis, a healthcare associated intestinal infection that mostly affects elderly patients with other underlying diseases. *Candida* species such as *C. albicans*, *C. parapsilosis* and *C. glabrata* may be detected. *Cryptococcus* species such as *C. neoformans* may be detected. Fungaemia
30 such as *Candidaemia* may be detected (presence or absence) using the invention. The microorganism is preferably (although this is not essential) indicated through its enzymatic activity. Thus, the methods provide an indication of viable, or recently so, microorganisms in the sample. After a period of time, if the microorganisms are not viable, the enzymatic activity would be lost from the sample. This represents an advantage of using enzymatic

activity as an indicator of microorganisms in the sample over use of nucleic acid molecules, in particular DNA, which may persist for much longer.

5 The methods of the invention may involve identifying the nature of the infection, once the positive presence of a microorganism has been detected in the sample. Any suitable method may be employed for this further identification step.

The invention also provides kits useful for performing the methods of the invention. Thus, there is further provided a kit comprising:

- 10 (a) a reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
(b) a first filter that prevents passage of insoluble cellular material through the filter
(c) a second filter that retains microorganisms within or upon the filter

15 All aspects and embodiments described in relation to the methods of the invention apply *mutatis mutandis* to the related kits.

In some specific embodiments, the kit further comprises

- (d) detection means for detecting the absence or presence of microorganisms retained
20 within or upon the filter.

Any suitable detection means may be employed and they may represent the complete set of reagents needed for detecting the absence or presence of microorganisms retained within or upon the filter in some embodiments.

25

In certain embodiments, the detection means comprises, or is, a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms.

30 In some embodiments, the detection means comprise or further comprise reagents for nucleic acid amplification. The reagents for nucleic acid amplification may comprise primers and/or probes. In some embodiments, those primers and/or probes hybridise to a microorganism nucleic acid molecule. They may therefore allow detection of microorganisms in the sample by detecting the amplified microorganism nucleic acid molecule. Alternatively, the primers or probes hybridise to a nucleic acid molecule which

acts as a substrate for nucleic acid modifying activity of the microorganisms. Such nucleic acid molecules are described in further detail herein.

Since in preferred embodiments the captured microorganisms are lysed in the filter, the kit
5 may further comprise a reagent capable of lysing microorganisms. Suitable reagents are described herein. This facilitates detection of the microorganisms.

In some embodiments, the kit comprises a nucleic acid molecule which acts as a substrate
10 for nucleic acid modifying activity of the microorganisms. This substrate may be included in the reagent capable of lysing microorganisms retained within or upon the filter. The substrate may be designed on the basis that the nucleic acid modifying enzyme comprises a DNA or RNA polymerase. In some embodiments, the DNA polymerase is DNA polymerase I. In additional or alternative embodiments, the nucleic acid modifying enzyme comprises a ligase, such as an ATP- or NAD-dependent ligase.

15 In certain embodiments, the reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample comprises a combination of a detergent and one or more enzymes. In certain embodiments, the one or more enzymes optionally comprise a proteinase and/or a DNase. Suitable detergents and
20 enzymes are discussed herein.

As already discussed, exposing the lystate to high pH is advantageous in the context of the present invention. Thus, in some embodiments, the kit further comprises a high pH
25 reagent. This may be a base or a buffer for example. This may, for example, be NaOH, e.g. 5mM NaOH. Suitable high pH reagents are discussed in greater detail elsewhere herein.

The kit may further comprise a neutralisation buffer to restore the pH of the sample
30 following the high pH treatment. Suitable reagents are discussed herein.

Suitable filters and dimensions thereof are discussed in greater detail in relation to the methods of the invention, which discussion also applies to the kits. Thus, the first filter included in the kits may comprise a(n average) pore size on the inlet side of at least 1 μm . In some embodiments, the (average) pore size on the inlet side is between 1 μm and 5 μm ,
35 such as between 1 μm and 2 μm .

In certain embodiments, the second filter comprises a(n average) pore size on the outlet side of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than 0.2 μm .

5

As discussed herein, the first and second filter may be provided as a single filter/filtration body comprising a pore size on the inlet side of the filter/filtration body that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter that retains microorganisms within the filter/filtration body. This may be achieved by a filter/filtration body comprising an asymmetric pore structure. In such embodiments, the pores decrease in size between the inlet and outlet sides of the filter/filtration body.

10

In other embodiments, the first and second filter are provided as a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter/filtration body.

15

According to all embodiments, the first and/or second filter may be a membrane. Suitable materials are discussed herein.

In further embodiments, the kit further comprises a filtration housing for housing the first and second filter. The housing may be provided in the kit with the filters provided separately. Alternatively, the kit may be provided with the filters pre-housed in the filtration housing. The filtration housing typically comprises an inlet and an outlet. In some embodiments, the inlet of the filtration housing is located relative to the outlet of the filtration housing such that a net direction of flow of fluid through the filtration housing is parallel to the plane of the first filter when housed in the filtration housing. In some embodiments, the housed second filter is in the same plane as the first filter and laterally offset within that plane. This provides advantages in the context of performing the methods of the invention. More specifically, the housed second filter may be in a parallel plane to the housed first filter.

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The invention also provides related devices, compositions of matter and kits. Thus, the invention provides a device, composition of matter or kit for detecting the absence or presence of a (viable or recently viable) microorganism in a sample that may also contain non-microorganism cells comprising:

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- a. a first reservoir for, or containing, a first lysis reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. a second reservoir for, or containing, a second lysis reagent that lyses microorganisms in the sample
- c. a sample inlet for addition of the sample to enable mixing of the sample with the first lysis reagent
- d. a filtration housing comprising:
 - I. a first filter which captures insoluble cellular material (which may include precipitated nuclear material)
 - II. a second filter, downstream of the first filter, which captures microorganisms
 - III. an outlet downstream of the second filter; and
- e. a fluid channel arrangement configured to provide selective fluid communication between the filtration housing and any one or more of the first reservoir, the second reservoir and the sample inlet.

All aspects and embodiments described in relation to the methods of the invention apply *mutatis mutandis* to the related devices, compositions of matter and kits.

In certain embodiments, the device, composition of matter or kit comprises a filtration housing inlet upstream of the first filter wherein the fluid channel arrangement is in fluid communication with the filtration housing inlet.

In certain embodiments, the device, composition of matter or kit comprises a mixing chamber in selective fluid communication with the sample inlet and the first reservoir.

The fluid channel arrangement may comprise one or more valves for providing the selective fluid communication.

In certain embodiments, the device, composition of matter or kit may comprise a filtration housing supplementary inlet downstream of the first filter and upstream of the second filter configured to be in selective fluid communication with the second reservoir so as to enable the second lysis reagent to be delivered directly to the second filter.

In specific embodiments, the device, composition of matter or kit comprises an asymmetric pore structure filter/filtration body that comprises the first filter and the second filter. In some embodiments, the pores of the asymmetric pore structure filter decrease in size between inlet and outlet sides of the filter.

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In certain embodiments, the device, composition of matter or kit comprises a laminate structure filter/filtration body having a first layer that comprises the first filter and a second layer that comprises the second filter.

10 According to all embodiments, the first filter may be a membrane. According to all embodiments, the second filter may be a membrane. Suitable membrane materials are discussed herein and include polysulfone membranes, such as polyethersulfone membranes (e.g. available from Merck Millipore). Other membrane materials that may be
15 Millipore) and glass fibre filters, optionally with a binder (e.g. available from Merck Millipore). Each may be provided in asymmetric form or as a laminate structure as discussed herein.

The first filter may comprise a minimum pore size on an inlet side of at least 1 μm . In some
20 embodiments, the first filter comprises a pore size on an inlet side of between 1 μm and 5 μm , such as between 1 μm and 2 μm .

The second filter may comprise a maximum pore size on an outlet side of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than
25 0.2 μm . A particularly suitable membrane type tested herein is an asymmetric polysulfone membrane with a pore size of around 0.22 μm (on the outlet side).

The device, composition of matter or kit may further comprise a mixing chamber in fluid communication with the first reservoir and comprising the sample inlet.

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The device, composition of matter or kit may further comprise one or more valves to control flow of fluid into the filtration housing.

The device, composition of matter or kit may further comprise one or more syringes and/or
35 pistons configured to transfer fluid from the first and/or second reservoirs into the filtration

housing. In some embodiments, the one or more syringes and/or pistons is configured to transfer fluid into the mixing chamber.

5 The device, composition of matter or kit may further comprise a fluid bath surrounding the filtration housing.

10 In some embodiments the inlet of the filtration housing is located relative to the outlet of the filtration housing such that a net direction of flow of fluid through the device or kit is parallel to the plane of the first filter. The second filter may be in the same plane as the first filter and laterally offset within that plane. The second filter may be in a parallel plane to the first filter.

15 The device, composition of matter or kit may further comprise a plinth configured to support the device or kit on a support plane in an orientation of use, and wherein the filtration housing projects in a direction perpendicular to the support plane. In some embodiments, the inlet of the filtration housing is located towards a lower edge of the filtration housing that is closer to the plinth and the outlet of the filtration housing is towards a higher edge of the filtration housing that is further from the plinth.

20 The invention also relates to a multi-channel device, composition of matter or kit comprising a plurality of the devices, compositions of matter or kits as described herein in parallel wherein the first and second filters of the plurality of the device or kit are located such that a net direction of flow of fluid through the multi-channel device or kit is parallel to the plane or planes of the first and second filters.

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DESCRIPTION OF THE FIGURES

Figure 1 is a perspective view of a single-channel cartridge POPI (SCP) with a dual filter configuration showing the reagent tank designations and valve positions

30 Figure 2 is a plan view of a SCP with a dual filter configuration showing the reagent tank designations and valve positions

Figure 3 shows a flow chart of the test process using the SCP.

Figures 4A to 4G are pressure vs. time plots for different filter configurations (A-G, following the order in Table 3) showing the points at which: Reagent A blood lysate reached the 0.22 μm filter (FF); the 5 mL pressure reading was measured; and the full quantity of Reagent A blood lysate had been pumped from the syringe.

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Figure 5 is a histogram showing WBC counts within patient population from Momentum's clinical validation study (n = 1472).

Figures 6A to 6E are pressure vs. time plots for shortlisted filter configurations (A-E, following the order in Table 4) showing the points at which: Reagent A blood lysate reached the 0.22 μm filter (FF); the 5 mL pressure reading was measured; and the full quantity of Reagent A blood lysate had been pumped from the syringe.

Figures 7A to 7E show test results for shortlisted filter configurations with (A) NSCs; (B) *E. coli*; (C) *S. aureus*; and (D-E) *C. albicans*. These tests were all performed with the 0.22 μm filter at 37°C for the entire process, whilst no heating was applied to the 5 μm filter in the dual filter configuration. Up to 16 x 100 μL fractions were collected and analysed by qPCR for single filter configurations, whereas up to 20 x 100 μL fractions were collected and analysed for the dual filter configuration to account for the volume added by the extra filter.

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Figures 8A and 8B show test results for different filter temperature conditions: 0.22 μm PES (single filter) with filter at room temperature for entire process (including 60 minutes incubation in LM) followed a 30 minute incubation of LM fractions at 37°C (PES (RT) on graph legends); 0.22 μm PES (single filter) with filter heated to 37°C for 60-minute LM incubation only (PES (37) on graph legends); and 5 μm PVDF + 0.22 μm PES (dual filter) with both filters heated to 37°C for the entire process (PVDF + PES (37T) on graph legends). Results are shown for (A) *C. albicans* and (B) NSCs. 16 x 100 μL LM fractions were collected and analysed by qPCR for single filter configurations, whereas 20 x 100 μL fractions were collected and analysed for the dual filter configuration to account for the volume added by the extra filter.

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Figure 9 shows a schematic representation of a first embodiment of a device, composition of matter or kit in accordance with the disclosure;

Figure 10 shows a schematic representation of a second embodiment of a device, composition of matter or kit in accordance with the disclosure;

Figure 11 shows an exterior of a filter assembly in accordance with the device, composition
5 of matter or kit of the first embodiment;

Figure 12 shows a cross-section through the filter assembly of Figure 11;

Figure 13 shows an exterior of a filter assembly in accordance with device, composition of
10 matter or kit of the second embodiment;

Figure 14 shows an interior of half of the filter assembly of Figure 13;

Figure 15 shows a cross-sectional view of an apparatus or cartridge including the filter
15 assembly of Figure 11 and other elements of the device, composition of matter or kit shown in Figure 9;

Figure 16 shows the apparatus or cartridge of Figure 15.

20 **SPECIFIC DESCRIPTION**

The schematic of Figure 9 relates to a first embodiment of a device, composition of matter or kit wherein the first and second filters 643, 644 are located, respectively, in first and second filter housings 641, 642 of the filter assembly 640.

25 The device, composition of matter or kit 600 comprises a first reservoir 510 for, or containing, the first lysis reagent and a second reservoir 520 for, or containing, the second lysis reagent. The device, composition of matter or kit 600 further comprises a sample inlet 621, a mixing chamber 620, a syringe 630, and an outlet 690 from which a fluid may be received into a receptacle 691. Furthermore, the device, composition of matter or kit 600
30 comprises a fluid channel arrangement comprising fluid channels 611, 612, 613, 614, 615, 616 and a plurality of valves 551, 552, 672, 673, 674, 675, 676. The syringe 630 allows for transfer of fluid from the first reservoir 510, the second reservoir 520 and the mixing chamber 620 into the filter assembly 640.

In addition, the device, composition of matter or kit 600 comprises an inlet filter assembly 622, containing a sample inlet filter 623, a mixing chamber vent 624, a fluid bath waste chamber 150 having a vent 151 and a thermal interface region 160.

- 5 The syringe 630 comprises a syringe housing 631, a syringe member 632, a syringe actuator 633 and a syringe access port 635. The syringe member 632 comprises a syringe member face 634 that may be surrounded by a circumferential O-ring or similar so as to provide an air-fluid-tight seal between the O-ring or similar of the syringe member face 634 and an interior of the syringe housing 631.

10

The syringe 630 and the various valves 551, 552, 672, 673, 674, 675, 676 may be controlled such that:

the first lysis reagent and the sample are mixed together in the mixing chamber 620;
the content of the mixing chamber 620 is transferred to the filter assembly 640

15

whereupon insoluble cellular material is captured on the first filter 641;

the second lysis reagent is transferred to the filter assembly 640; and
microorganisms are captured on the second filter 642.

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The schematic of Figure 10 relates to a second embodiment of a device, composition of matter or kit wherein the first and second filters 643' are located in a single filter housing 641' of the filter assembly 640'.

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In this embodiment, first and second filters 643' may be formed of a single filtration body having a pore size on the inlet side of the filtration body that prevents passage of insoluble cellular material and a pore size on the outlet side of the filtration body that retains microorganisms within the filtration body.

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Such a filtration body may comprise an asymmetric pore structure. Thus, the filtration body may comprise pores that (gradually) decrease in size between the inlet and outlet sides of the filtration body.

In an alternative arrangement in accordance with the second embodiment of the device, composition of matter or kit, the first and second filters may be formed through a laminate structure. Thus, in some embodiments, the two filtration steps are performed using a filter

comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter.

Shown in Figure 11 is an example of a filter assembly 640 in accordance with the first
5 embodiment of the device, composition of matter or kit shown in Figure 9. The filter
assembly 640 comprises a first portion 645 containing the first filter housing 641 and a
second portion 646 containing the second filter housing 642. The filter assembly 640 is
formed from a first side 647 and a second side 648. The first filter housing 641 and the
second filter housing 642 are formed between the first side 647 and the second side 648.

10 Figure 12 shows a cross-section through the filter assembly of Figure 11. The first filter
housing 641 and the second filter housing 642 each comprise a collection of recesses and
protrusions 665, 667, 668, 669 by which a filter may be retained in position within the
respective housing 641, 642. The collection of recesses and protrusions 665, 667, 668,
15 669 may also serve to encourage flow of fluid in a preferred direction within the respective
housing 641, 642.

Figure 13 shows an example of a filter assembly 640' in accordance with the second
embodiment of the device, composition of matter or kit shown in Figure 10. The filter
20 assembly 640' is formed from a first side 647' and a second side 648'. The filter housing
641' is formed between the first side 647' and the second side 648'.

Figure 14 shows the second side 648' of the filter assembly 640' of Figure 13. As in the
Figure 12 example, protrusions and recesses are provided within the filter housing 641' for
25 the same purposes as in the Figure 12 arrangement.

Figure 15 shows a cross section through an apparatus or cartridge 100 including a plurality
of processing channels, each channel comprising one of the filter assembly 640 of Figure
11. The apparatus or cartridge 100 also comprises other elements of the device,
30 composition of matter or kit shown in Figure 9 including the second reservoir 520, the
syringe 630 fluid bath waste chamber 150. The apparatus or cartridge 100 further
comprises the first reservoir (not visible in Figure 15). In this embodiment, a single first
reservoir and a single second reservoir supply all of the plurality of channels. Similarly, a
single fluid bath waste chamber 150 is provided to serve all channels.

Figure 16 shows apparatus or cartridge 100 from which it can be seen that, in this example, the apparatus or cartridge 100 comprises eight channels.

5 The apparatus or cartridge 100 comprises a housing 101 and a plinth 102 located at a perimeter of a lower surface of the filter cartridge housing 101 on which the apparatus or cartridge 100 is supported when resting on a planar surface in the orientation shown in Figure 16. The apparatus or cartridge 100 comprises valve actuators 111, 112, 113, 114, 115, 116 for controlling the valves 551, 552, 672, 673, 674, 675, 676 shown in Figure 9. The valve actuators 111, 112, 113, 114, 115, 116 (at least when in an inmost position) are
10 located within recesses in the plinth 102 so as to provide protection. Similarly, the syringe actuators 633 (one for each channel) are each located within the boundaries of the plinth 102, at least when in an inmost position.

A similar apparatus or cartridge (not illustrated) may be provided to comprise one or more
15 channels each comprising the filter assembly 640' having only a single filter housing 641' as shown in Figure 13. Other features of the apparatus or cartridge 100 may be substantially the same as in the illustrated embodiments of Figures 15 and 16.

Whether the apparatus or cartridge 100 has a single filter housing 641' (per channel) or first
20 and second filter housings 641, 642 (per channel), the apparatus or cartridge 100 provides or is capable of providing the functionality of the device, composition of matter or kit to facilitate the method of separating microorganisms from non-microorganism cells. Furthermore, in the illustrated apparatus or cartridge 100, eight separate samples may be separated simultaneously in eight independent channels. A multi-channel apparatus or
25 cartridge having a number of channels other than eight falls within the scope of the disclosure. Furthermore, multi-channel functionality is an optional feature of the apparatus or cartridge and an apparatus or cartridge having only single channel functionality falls within the scope of the disclosure.

30 The invention will be understood with respect to the following non-limiting examples:

EXPERIMENTAL SECTION

EXAMPLE 1 - SINGLE VS. DUAL FILTER DESIGN

5 1 **Purpose**

To determine whether one filter housing or two filter housings are required for the eight-channel Cognitor Minus HT cartridge. The following criteria were assessed:

- Filtration performance for 'normal' and 'elevated nucleated cell number' blood samples
- 10 • ETGA qPCR results for negative (sterile blood) and positive samples (microorganism-spiked blood)

2 **Introduction**

The filter element of the Cognitor Minus HT disposable cartridge must allow filtration of
15 Reagent A lysed blood broth; whilst capturing and immobilising any intact microorganisms that are present. Reagent A utilises a detergent-based approach to lysing blood cells with the aid of Proteinase K to further break up cellular debris and chromatin. Microorganisms are not lysed by Reagent A because of their cell wall. After filtration of Reagent A blood lysate the filter must allow passage of Reagent B (5 mM NaOH) to denature and wash
20 away lysed blood material; followed by passage of Reagent C (neutralisation buffer) to neutralise alkali conditions and provide further washing of the filter membrane. After passage of Reagent C, the filter membrane should contain only immobilised microorganisms. The filter element is then filled with Lysis Mix (LM) under temperature controlled conditions, resulting in the enzymatic lysis of any microorganisms that are
25 present and the subsequent generation of ETGA template DNA by microorganism-derived DNA polymerase.

The aim of the work described here was to determine whether one filter housing or two filter housings are required for optimal functioning of the Cognitor Minus HT cartridge. The
30 filter element of the cartridge must allow filtration of lysed blood broth specimens without blocking. The ability to pass lysed blood broth through a filter membrane is effected by the number of nucleated blood cells present due to differences in the amount of chromatin which is released and can precipitate upon lysis of the nuclear membrane. Whilst healthy adult human blood contains mostly non-nucleated red blood cells (approximately 45% of
35 whole blood) with less than 1% nucleated white blood cells (WBC) (normal range for

healthy adults: 5 to 15 million WBC/mL), our clinical validation study (ETGA Minus VV PHASE 2 2015) revealed that clinical blood specimens contain up to 500 million WBC/mL.

The Cognitor Minus HT test must be capable of filtering clinical blood specimens with elevated WBC counts. The filter membrane should have a pore size no larger than 0.2 µm so that it can provide universal microorganism capture. Our key design approach for filtration of lysed blood broth was to prevent blockage of 0.2 µm pores by holding back insoluble lysis material using an upstream barrier with a larger pore size. The following filter configurations were tested:

- A single membrane with 0.22 µm pores (0.22 µm PVDF)
- A single asymmetric membrane with large pores on the inlet-side gradually decreasing in size to 0.22 µm pores on the outlet side (0.22 µm PES)
- The addition of a glass fibre pre-filter (1 µm GF or 2 µm GF) in front of a 0.22 µm pore-size membrane (0.22 µm PVDF or 0.22 µm PES) within the same filter housing
- The addition of a separate upstream filter housing containing a membrane with larger pores (5 µm PVDF)

The experiments described in this report are separated into three sections:

1. Filtration performance of different filter configurations using 'normal' sheep blood preparations in order to identify a shortlist for further testing
2. Filtration performance of three shortlisted filter configurations using sheep blood supplemented with nucleated chicken blood cells to simulate filtration of clinical blood specimens with elevated WBC counts
3. Cognitor Minus HT test performance for negative (sterile blood) and positive (microorganism-spiked blood) samples using the single-channel cartridge POPIs (SCPs)

3. Materials and Methods

3.1 General Reagent Compositions

Reagent A

0.25% Triton X-100

4.8 µg/mL Proteinase K

Reagent B

5 mM Sodium hydroxide

Reagent C

- 5 10 mM Ammonium sulphate
2 mM Magnesium sulphate heptahydrate
10 mM Potassium chloride
20 mM Tris-HCl [pH 8.0]

- 10 Lysis Mix (LM)
Comprised of L1 (P005), L2, L3 (P007), dNTPs, PTO-IPC stock (P014)

L1: 252 mL in 360 mL LM

1.46% (w/v) BSA

- 15 0.15% Triton X100
0.15% Tween 20

L2: 36 mL in 360 mL LM

100 mM Ammonium sulphate

- 20 20 mM Magnesium sulphate heptahydrate
100 mM Potassium chloride
200 mM Tris-HCl [pH 8.0]

L3: 36 mL in 360 mL LM

- 25 0.1 μ M PTO-AS oligo
0.1 μ M PTO-S1 oligo
20 mM Tris-HCl [pH 8.5]
10 mM Potassium chloride
10 μ M EDTA

- 30
10 mM dNTPs: 3.6 mL in 360 mL LM

PTO-IPC stock: ~180 μ L in 360 mL LM (*Note: variable concentration)

- 35 H₂O: ~32.4 mL in 360 mL LM

3.2 Filtration performance methods

- Reagent A blood lysis preparation for filtration performance with 'normal' blood:
 - o 5 mL BacT/ALERT SA broth (BioMerieux) supplemented with sheep blood (Sheep Blood in Alsever's adjusted to approximately 50% PCV by removal of settled supernatant; TCS Biosciences Cat# SB069 Lot# 30523900) was added to 7.5 mL Reagent A (working concentrations: 0.2% Triton X-100, 3.84 µg/mL Proteinase-K) and incubated for 20 minutes at room temperature (approximately 20°C).
- Reagent A blood lysis preparation for filtration performance with 'elevated nucleated cell number' blood:
 - o Haemocytometer cell counts were performed for a suspension of chicken blood cells (Chicken Blood Cells in Alsever's adjusted to approximately 40% PCV by removal of settled supernatant; TCS Biosciences Cat# FB011AP Lot# 30599900).
 - o Chicken blood cells were added to BacT/ALERT SA broth supplemented with sheep blood (Sheep Blood in Alsever's adjusted to approximately 50% PCV by removal of settled supernatant) in a total volume of 2.5 mL (see Table 1) and immediately added to 10 mL Reagent A (working concentrations: 0.2% Triton X-100, 3.84 µg/mL Proteinase-K), and incubated for 20 minutes at room temperature (approximately 20°C).

Table 1: Preparation of blood broth samples with elevated nucleated blood cell numbers

Nuclei/mL in blood broth	Equivalent to WBC/mL in blood	Sample vol. (µL)	Vol. sheep blood broth (µL)	Vol. chicken blood (µL)	% Patients ≤WBC
100 × 10 ⁶	500 × 10 ⁶	2500	2394	106	100.0
50 × 10 ⁶	250 × 10 ⁶	2500	2447	53	99.9
20 × 10 ⁶	100 × 10 ⁶	2500	2479	21	99.6

% Patients ≤WBC: proportion of patients from clinical validation study (n = 1472) with WBC counts lower than or equal to the number of nucleated chicken blood cells stated

- For single filter configurations 10 mL Reagent A blood lysate was pumped through the Filter POPI using a syringe pump (Cole-Parmer RZ-74900-35) set at a flow rate of 1 mL/min. This was followed with 10 mL Reagent B by syringe pump (1 – 2 mL/min) or manual pumping. For dual filter configurations 11 mL Reagent A and Reagent B were pumped from the syringe to account for approximately 1 mL volume added by the upstream filter housing.

- Filtration was performed using 0.8 mm ID tubing and the following filter membranes cut to a diameter of 26 mm (22.5 mm after clamping):
 - o 0.22 µm filter membranes
 - DURAPORE PVDF 0.22 µm (Merck Millipore, ref GVWP04700): 0.22 µm PVDF
 - Asymmetric Express PLUS PES 0.22 µm (Merck Millipore, ref GPWP04700): 0.22 µm PES
 - o Glass fibre pre-filters (patterned side facing inlet)
 - Glass Fibre Filter with binder 1.0 µm (Merck Millipore, ref AP1504200): 1 µm GF
 - Glass Fibre Filter with binder 2.0 µm (Merck Millipore, ref AP2004200): 2 µm GF
 - o 5 µm filter membrane
 - DURAPORE PVDF 5.0 µm (Merck Millipore, ref SVLP04700): 5 µm PVDF
- A digital manometer was used to record pressure (PSI) versus time (seconds). Pressure values at the point of filtering a given volume of Reagent A blood lysate are annotated to enable quantitative comparison of filtration performance.
- Approximately 0.5 mL fluid in feed tube (with an additional 0.5 mL - 1 mL fluid for dual filter configurations); and up to 0.5 mL fluid lost to the manometer tube.

3.3 Cognitor Minus HT test performance methods

BacT/ALERT blood broth (Sheep Blood in Alsever's; TCS Biosciences Cat# SB069 Lot# 30523900) was inoculated to approximately 1×10^7 cfu/mL for *E. coli* and *S. aureus*; and approximately 1×10^5 cfu/mL for *C. albicans*. TVC plates were prepared for inoculated blood broth samples (Columbia Agar Base for *E. coli* and *S. aureus*; and Sabouraud Dextrose Agar for *C. albicans*) and 'No Spike Controls' (NSCs) (Columbia Agar Base). 1 mL blood broth was added to the mixing chamber of each SCP. 4.5 mL Reagent A, 5.5 mL Reagent B, 5.5 mL Reagent C and 3.2 mL - 4 mL LM were added to their respective reagent tanks on each SCP (see Figures 1 and 2). The compositions of Reagent A and LM differed for different microorganisms depending on their individual lysis requirements (see Table 2).

Table 2: Reagent compositions for different test microorganisms and controls

Microorganism/ control	Reagent A additives (working conc.)	LM composition (working conc.)
<i>E. coli</i> (ATCC 29522)	None	0.01 mg/mL Lysozyme (Sigma L3790), 0.005 mg/mL Polymyxin B (Sigma 92283)
<i>S. aureus</i> (ATCC 29523)	None	0.002 mg/mL Lysostaphin (Abcam ab70102)
<i>C. albicans</i> (ATCC 10231)	8 mM DTT	100 KU/mL Lyticase (Sigma L2524)
No Spike Control	None	None

The Cognitor Minus HT test was carried out according to the process described in Figure 3. After manual pumping of Reagent A into the mixing chamber to purge air from the system, all reagents were pumped through the filter(s) at 4 mL/min using a syringe pump (Cole-Parmer RZ-74900-35). A heater element on the outlet side of the filter was heated to a set point of 44.5°C to give a fluid temperature of 37°C +/- 1°C inside the filter housing (time to reach 37°C was 5 to 10 minutes). The majority of tests were performed with the heater element switched on from the start, however some tests investigated the effect of filter temperature (see results section for individual test details). The volume of the system from syringe to output/waste valve (V5/V6) is approximately 1.2 mL for single filter configurations and approximately 2.2 mL for dual filter configurations. To identify which LM fraction(s) had the highest concentration of ETGA template DNA, 100 µL fractions of LM were collected at a flow rate of 2 mL/min and tested by qPCR. Where appropriate, LM negative and positive controls were performed by incubating 10 µL LM in a tube with or without DNA polymerase I on a 37°C hot block for 60 minutes. After each test, SCPs were decontaminated using 70% 2-Propanol and H₂O.

4 Results and discussion

4.1 Filtration performance with 'normal' blood

In order to rigorously test a variety of filter configurations a blood broth to lysis buffer ratio of 2:3 in a total volume of 10 mL (i.e. 4 mL blood culture + 6 mL Reagent A) was pumped through each filter configuration from an upstream syringe. 11 mL Reagent A blood lysate was pumped for dual filter configurations to account for volume added by an extra filter housing. Whilst the Cognitor Minus HT test is currently intended to process 1 mL blood broth in a total volume of 5 mL with Reagent A; a larger proportion of blood broth in a larger total volume was tested with the aim of pushing each filter configuration to maximum capacity to allow easier differentiation of their filtration performance.

The results for filtration performance using different filter configurations are shown in Table 3 and Figure 4. The columns showing pressure values for the points when either 5 mL or 9 mL Reagent A blood lysate had passed the fine filter (0.22 μ m pore-size) are colour coded (red to green for low to high pressure respectively) to enable easier comparison of filtration performance. All filter configurations were capable of filtering the full volume of Reagent A blood lysate apart from '0.22 μ m PVDF' which could only pass 3.80 mL before fluid entered the manometer due to high pressure (final pressure of 6.20 PSI). The '0.22 μ m PVDF' single filter could only pass an additional 1 mL Reagent A blood lysate with the manometer detached before filter blockage occurred. The full volume of Reagent B was successfully filtered by all filter configurations, including '0.22 μ m PVDF' despite filter blockage by Reagent A blood lysate. However, some filter configurations required manual pumping to prevent fluid entering the manometer when pressure had become too high during passage of Reagent A blood lysate.

The pressure values demonstrate that '0.22 μ m PES' provided the best filtration performance with a 5 mL pressure value of 1.19 PSI and 9 mL pressure value of 2.55 PSI. The glass fibre pre-filter (2 μ m and 1 μ m pore-size) in combination with 0.22 μ m PES or 0.22 μ m PVDF provided the next best filtration performance with 5 mL pressure values ranging from 1.72 PSI to 2.10 PSI and 9 mL pressure values ranging from 2.63 PSI to 2.94 PSI. The '5 μ m PVDF + 0.22 μ m PES' dual filter configuration had a 5 mL pressure value of 1.81 PSI, which is comparable to the glass fibre pre-filter filter configurations. However, pressure continued to increase at a faster rate after this point resulting in a 9 mL pressure value of 3.45 PSI. Excluding '0.22 μ m PVDF', the '5 μ m PVDF + 0.22 μ m PVDF' dual filter configuration provided the worst filtration performance with a 5mL pressure value of 2.71 PSI and a 9 mL pressure value of 5.55 PSI.

During testing air bubbles were observed for certain filter configurations (see 'Notes' column in Table 3). This was due to an inability to pass air through a wet filter membrane, which can cause air bubbles to become trapped on the inlet side of the filter when liquid bleeds through the membrane before the fluid has completely filled the inlet side of the filter housing. Trapped air bubbles reduce the surface area available for filtration, and hence reduce filtration performance. Air traps can be reduced by increasing flow rate and by reducing the internal volume of the filter housing inlet.

Table 3: Filtration performance with 'normal' blood

Filter configuration	Pressure at 5 mL (PSI)	Pressure at 9 mL (PSI)	Notes
0.22 µm PVDF (single filter)	*6.20	N/A	RA stopped after passing 3.8 mL through filter due to fluid entering manometer; only 1 mL extra RA could pass through manually with manometer detached
0.22 µm PES (single filter)	1.19	2.55	10% air on filter
1 µm GF + 0.22 µm PVDF (single filter)	1.72	2.63	N/A
2 µm GF + 0.22 µm PVDF (single filter)	1.79	2.74	N/A
2 µm GF + 0.22 µm PES (single filter)	2.10	2.94	N/A
5 µm PVDF + 0.22 µm PVDF (dual filter)	2.71	5.55	20% air on 5 µm filter
5 µm PVDF + 0.22 µm PES (dual filter)	1.81	3.45	20% air on 5 µm filter; and 15% air on 0.22 µm filter

*Pressure value at point when 3.80 mL Reagent A blood lysate had passed through filter. RA: Reagent A

Based on these results three filter configurations were shortlisted for further testing (see sections 4.2 and 4.3):

- 0.22 µm PES (single filter)
- 2 µm GF + 0.22 µm PVDF (single filter)
- 5 µm PVDF + 0.22 µm PES (dual filter)

The '0.22 µm PES' filter was shortlisted because it had the best filtration performance of all filter configurations tested. '2 µm GF + 0.22 µm PVDF' was selected, despite performing similarly to '1 µm GF + 0.22 µm PVDF', because previous testing with a blood broth to lysis buffer ratio of 1:4 showed that '2 µm GF + 0.22 µm PVDF' provided slightly lower filtration pressures (data not shown). The '5 µm PVDF + 0.22 µm PES' dual filter configuration was shortlisted, despite performing worse than certain GF pre-filter configurations, because it was deemed important to compare both single and dual filter configurations in the context of the full Cognitor Minus HT test (see section 4.3).

4.2 Filtration performance with 'elevated nucleated cell number' blood

To test the ability of the shortlisted filter configurations to filter blood samples containing an elevated number of nucleated blood cells, sheep blood broth (adjusted to approximately 50% PCV before dilution in broth: comparable to human blood with a PCV of approximately 45%) was supplemented with different quantities of chicken blood cells to simulate clinically relevant WBC counts (see Table 1). As '0.22 µm PES' provided the best filtration performance for 'normal' blood, this filter configuration was used to ascertain a suitable nuclei/mL level for comparison of filtration performance across the full shortlist. The

nuclei/mL levels tested were intended to simulate the highest WBC counts observed in clinical specimens from Momentum's clinical validation study (see Figure 5).

5 The results for filtration performance with 'elevated nucleated cell number' blood are shown in Table 4 and Figure 6. Blood broth with 100×10^6 nuclei/mL (equivalent to 500×10^6 WBC/mL: the highest WBC count observed in clinical validation study) appeared to lyse effectively upon addition of Reagent A (clear lysate with no visible insoluble material), but caused complete filter blockage after passing only 2.90 mL through the filter resulting in a final pressure value of 8.09 PSI. Blood broth with 50×10^6 nuclei/mL (equivalent to 250×10^6 WBC/mL: 99.9 % of specimens in clinical validation study had a WBC count lower than or equal to this value) appeared to lyse effectively upon addition of Reagent A (clear lysate with no visible insoluble material), but could only filter 5.35 mL before high pressure caused fluid to enter the manometer. However, the full 10 mL Reagent A blood lysate was successfully filtered by manual pumping with manometer detached. Pumping of Reagent B was not attempted for 100×10^6 nuclei/mL or 250×10^6 nuclei/mL blood broth lysates.

20 The '0.22 μ m PES' single filter configuration was able to filter the full 10 mL of lysed blood broth with the manometer connected for 20×10^6 nuclei/mL (equivalent to 100×10^6 WBC/mL: 99.6 % of specimens in clinical validation study had a WBC count lower than or equal to this value). Therefore, all shortlisted filter configurations were tested with this quantity of nucleated blood cells. '0.22 μ m PES' provided the lowest filtration pressure throughout passage of Reagent A blood lysate, with 5 mL and 9mL pressure values of 1.41 PSI and 5.21 PSI respectively. '2 μ m GF + 0.22 μ m PVDF' provided similarly low filtration pressure with 5 mL and 9 mL pressure values of 2.29 PSI and 5.97 PSI respectively; and was also capable of filtering the full 10 mL Reagent A blood lysate without fluid entering the manometer due to high pressure. The '5 μ m PVDF + 0.22 μ m PES' dual filter produced notably higher filtration pressure compared to the single filter configurations, with a 5 mL pressure value of 5.64 PSI. Furthermore, pumping had to be suspended after passing only 5.97 mL through the 0.22 μ m filter due to fluid entering the manometer. However, the full 11 mL of Reagent A blood lysate was successfully filtered by manual pumping with the manometer detached. The full volume of Reagent B was successfully filtered by all filter configurations, however manual pumping was required to prevent fluid entering manometer when pressure had become too high during Reagent A passage.

Table 4: Filtration performance with 'elevated nucleated cell number' blood

Filter configuration	Nuclei/mL in blood broth	Pressure (PSI) at 5 mL	Pressure (PSI) at 9 mL	Notes
0.22 μ m PES (single filter)	100 x 10 ⁶	*8.09	N/A	RA stopped after passing 2.90 mL through filter due to fluid entering manometer; no extra RA could pass through manually with manometer detached; 10% air on filter
0.22 μ m PES (single filter)	50 x 10 ⁶	7.76	N/A	RA stopped after passing 5.35 mL through filter due to fluid entering manometer; remaining RA successfully pumped through manually with manometer detached
0.22 μ m PES (single filter)	20 x 10 ⁶	1.41	5.21	10% air on filter
2 μ m GF + 0.22 μ m PVDF (single filter)	20 x 10 ⁶	2.29	5.97	N/A
5 μ m PVDF + 0.22 μ m PES (dual filter)	20 x 10 ⁶	5.64	N/A	RA stopped after passing 5.97 mL through 0.22 μ m filter due to fluid entering manometer; remaining RA successfully pumped through manually with manometer detached

*Pressure value at 2.90 mL Reagent A blood lysate through filter. RA: Reagent A

4.3 Cognitor Minus HT test performance

- 5 In addition to filtration performance it was deemed important to assess the performance of different filter configurations in the context of the full Cognitor Minus HT test. The key performance indicators considered were: the ability to remove blood-derived ETGA qPCR signal; and the strength of microorganism-derived ETGA qPCR signal. These factors indicate successful removal of blood material; and effective capture and lysis of
- 10 microorganisms for maximal ETGA template DNA production. Please note that the results presented here are only semi-quantitative due to the non-sterile environment of the SCPs. All tests were performed using the same batches of each reagent to limit reagent variability between tests that were not performed at the same time. Each of the graphs in Figure 7 and Figure 8 show data obtained from three tests performed in parallel.

15

4.3.1 Cognitor Minus HT results with 0.22 μ m filter at 37°C throughout

The Cognitor Minus HT results for samples processed with the 0.22 μ m filter heated to 37 °C for the entire process are shown in Figure 7. Note that the 5 μ m PVDF filter in the dual filter configuration was not heated at any point during the Cognitor Minus HT test for this

20 set of experiments.

The results for NSCs (Figure 7A) demonstrate that blood-derived ETGA signal (and/or system contamination-derived ETGA signal) was minimal across all LM fractions for all three filter configurations. Ct values for NSCs ranged from 41.07 Ct units to 'No Ct' and

25 there were no apparent trends in ETGA signal strength for different LM fractions. This low

level of background signal was reproducible during previous testing with the '0.22 μ m PES' single filter configuration (data not shown). Furthermore, comparison of test results for *E. coli* in broth and *E. coli* in blood broth demonstrated highly similar Ct values across all sixteen 100 μ L LM fractions (data not shown), indicating that blood is not contributing to the overall ETGA qPCR signal observed for microorganism-spiked blood broth samples.

In general, the results show that for microorganism-spiked blood broth samples two possible regions of strong ETGA signal were observed across the LM fractions: 300 μ L (400 μ L point on graph) to 700 μ L LM (observed for single and dual filter configurations); and 900 μ L (1000 μ L point on graph) to 1600 μ L (only observed for the dual filter configuration). These optimal regions are believed to correspond to the LM fractions adjacent to the microorganisms immobilised on the 0.22 μ m filter and 5 μ m filter respectively: smaller microorganisms (e.g. *E. coli*) are captured on the 0.22 μ m filter, whilst larger microorganisms (e.g. *C. albicans*) are captured on the 5 μ m filter.

The results for *E. coli* (Figure 7B) show that all three filter configurations performed equally well in terms of maximum ETGA signal, with the lowest Ct values ranging from 29.30 Ct units to 29.81 Ct units across the three filter configurations. The results for *S. aureus* (Figure 7C) also demonstrate that all three filter configurations produced similar Ct values within the optimal LM region surrounding the 0.22 μ m filter (lowest Ct values ranging from 32.74 Ct units to 34.49 Ct units across the three filter configurations). However, the lowest Ct values achieved with the '5 μ m PVDF + 0.22 μ m PES' dual filter configuration were within the optimal LM region surrounding the 5 μ m filter, with a lowest Ct value of 31.42 Ct units for LM fraction '1200 μ L to 1300 μ L'. A similar distribution of ETGA signal was observed for *C. albicans* (Figure 7D-E), with two experimental replicates showing much stronger ETGA signal in the optimal LM region surrounding the 5 μ m filter compared to the 0.22 μ m filter. Furthermore, the lowest Ct values observed for the dual filter configuration were much lower (stronger ETGA signal) compared to the single filter configurations, with up to 4.37 Ct units difference between the dual and single filter configurations. Due to the larger size of *C. albicans*, it was predicted that this microorganism would be captured primarily on the 5 μ m filter of the dual filter configuration, and therefore it was expected that the LM adjacent to this filter would produce the strongest ETGA signal. However, it was not expected that such a large difference in ETGA signal would be observed between the single and dual filter configurations. Assuming efficient capture of microorganisms and similar behaviour of reagents on the different filters, the most obvious dissimilarity between

the 5 µm filter (dual filter configuration only) and the 0.22 µm filters is the fact that the 5 µm filter was not heated during these experiments. Previous experiments using mechanical microbial lysis (bead milling) have demonstrated that the efficiency of the ETGA reaction is not improved by reducing the temperature to 34°C or 20°C for *E. coli*, *S. aureus* or *C. albicans*. Therefore, it was hypothesised that the lower temperature inside the 5 µm filter may be altering the microbial lysis efficiency of LM or the toxicity of other reagents to the microorganisms. Both of these scenarios could cause a reduction in the amount of microbial DNA polymerase available for ETGA template DNA production. To test this hypothesis the following Cognitor Minus HT tests were performed using blood broth spiked with *C. albicans*:

1. 0.22 µm PES (single filter) at room temperature throughout (including the 60 minute incubation with LM) followed by a 30 minute incubation of LM fractions at 37°C for the ETGA reaction to occur: This was to test whether the ETGA signal achieved with a single filter configuration can be increased by lowering the filter temperature.

2. 0.22 µm PES (single filter) at 37°C only during the 60 minute incubation with LM: This was to test whether ETGA signal is effected by the temperature of the filter during microbial capture and washing as an indicator for reagent toxicity.

3. 5 µm PVDF + 0.22 µm PES (dual filter) with both filters heated to 37°C throughout: This was to test whether ETGA signal is reduced when the temperature of the 5 µm filter is increased to 37°C.

4.3.2 The effect of filter temperature on Cognitor Minus HT results

The Cognitor Minus HT results for different filter temperature conditions are shown in Figure 8. Importantly, the TVC cfu/mL values for all three *C. albicans* experiments (Figure 7D, Figure 7E and Figure 8A) were very similar. Test results for blood broth spiked with *C. albicans* (Figure 8A) demonstrate that heating the 0.22 µm PES filter to 37°C only during the 60 minute LM incubation (PES (37) on graph in Figure 8A) had no apparent effect on ETGA signal (or the distribution of ETGA signal across LM fractions) compared to heating the filter to 37°C for the entire process (based on Ct values observed in Figure 7E). This result indicates that heating the filter to 37°C for the entire process does not alter the toxicity of reagents A, B or C to *C. albicans*. The ETGA signal achieved by the dual filter

configuration was dramatically reduced by heating both filters to 37°C throughout the test (PVDF + PES (37T) on graph in Figure 4 5A), with a lowest Ct value of 37.76 Ct units compared to 32.63 Ct units for the dual filter configuration without heating of the 5 µm filter (Figure 4 4D). Performing the test with the 0.22 µm PES single filter configuration at room temperature throughout (including the 60 minute LM incubation) with a 37°C ETGA reaction at the end greatly improved ETGA signal (PES (RT) on graph in Figure 8A). This condition achieved a lowest Ct value of 31.84 Ct units compared to 35.80 Ct units (ΔCt value of 3.96 Ct units) for a duplicate sample processed with the filter heated to 37°C during the 60 minute LM incubation (PES (37)). Importantly, the results for the same test conditions performed with NSCs (Figure 8B) demonstrate that these changes in filter temperature conditions have no effect on the amount of blood-derived ETGA signal. Therefore the observed differences in ETGA signal can be attributed to temperature effects. Taken together these results indicate that filter temperature has a strong effect on the lysis of *C. albicans* and potentially other microorganisms.

4.4 Conclusions

The results presented in this report demonstrate that the shortlisted single filter configurations ('0.22 µm PES' and '2 µm GF + 0.22 µm PVDF') are capable of providing better filtration performance and Cognitor Minus HT results compared with the best dual filter configuration ('5 µm PVDF + 0.22 µm PES'). However, both formats are better than use of a single filter that only captures the microorganisms and does not account for insoluble lysed material.

Both of the shortlisted single filter configurations were capable of filtering larger than required quantities of 'normal' blood at low pressure (less than 3.00 PSI). They were also capable of filtering blood containing a quantity of nucleated blood cells greater than 99.6% of clinical blood specimens from Momentum's clinical validation study (n = 1472; Figure 4 2). When performing the Cognitor Minus HT test, both single filter configurations were able to adequately remove blood-derived ETGA signal (based on negative blood samples) and produce strong microbial ETGA signal for positive samples.

Interestingly, altering filter temperature conditions for blood broth spiked with *C. albicans* had a strong effect on the amount of ETGA signal achieved with both the single and dual filter configurations. This observation indicates that the optimal temperature for the ETGA

reaction (37°C) is probably not the same as the optimal temperature for enzymatic microbial lysis.

In addition to superior filtration performance and Cognitor Minus HT results, an eight-channel cartridge with a single filter configuration also has other advantages over a dual filter configuration. Firstly, it reduces the volume of LM required to fill the system during the ETGA reaction, thereby reducing the potential for dilution of ETGA template DNA (ETGA qPCR signal). Secondly, it reduces the overall size of the cartridge making it more user friendly; and reduces the number of parts required to build the cartridge making it cost less to produce.

EXAMPLE 2 – DETECTION OF CLINICALLY RELEVANT SPECIES

1 Methods and materials

1.1 Equipment

1.1.1 Qiagen Rotor-Gene Q

30 µL reactions performed using 'Cognitor Minus V1.0' programme. Data analysis performed using the following Ct threshold settings:

- FAM threshold = 0.03 with slope correction
- ROX threshold = 0.1

1.2 General methods

1.2.1 Microbial strains

Where possible, the microorganism strains used in this work package were clinical strains isolated from I.D confirmed monomicrobial blood culture specimens on agar slopes (room temperature storage) at Basingstoke Hospital (UK), which were plated from individual colonies by Momentum Bioscience Ltd, incubated o/n at 37°C, grown in liquid culture o/n and then sub-aliquoted into glycerol stocks (3 parts LC to 1 part 80% glycerol; stored at -80°C). Experiments were performed using culture plates that were less than 1 week old after plating from glycerol stocks.

1.2.2 Human blood source

Human blood was supplied by Cambridge Bioscience in anti-coagulant vacutainers (K2-EDTA or Sodium Citrate) or collected directly into BacT ALERT SA bottles, and delivered on an overnight delivery (shipped at approximately 4°C).

5 1.2.3 Specimen preparation

Blood broth was pre-warmed for 30 minutes at 37°C before use. For microorganism-spiked blood broth specimens, overnight (o/n) liquid cultures were diluted to the desired cell density in appropriate liquid media and then added to blood broth (6.25 µL diluted o/n culture per 1 mL blood broth). Spiked and no spike control (NSC) blood broth specimens
10 were either used immediately or pre-incubated for a specified amount of time to allow microorganisms to enter exponential growth phase. Total viable counts (TVCs) were performed by spreading 100 µL of each specimen on appropriate agar plates at the beginning of each test.

2 Filtration Capture

15 Filter units according to the invention (and described in detail herein) were made from ultrasonically welded injection-moulded filter housing parts with an asymmetric polyether sulfone membrane (Merck Millipore, Express PLUS 0.22 µm filter membranes; Product# GPWP04700PES). This was the best performing membrane type from Example 1.

20 The aims of the filtration capture feasibility study were to demonstrate that:

1. Blood ETGA signal can be consistently eliminated for human blood broth specimens
2. Clinically-relevant microorganism species can be detected at approximately 10³ cfu/mL.

25

Clinically-isolated strains representing the 12 highest ranking genera from the Public Health England 2015 report for monomicrobial infection were tested at a single cell density (target cell density approximately 1000 cfu/mL), along with a no spike control (NSC) sample with each test run. Microorganisms were spiked into human blood broth and pre-incubated for a
30 specified amount of time with the aim of achieving exponential microbial growth. Different fractions of lysis mix (LM) sample output were collected and analysed by qPCR to ascertain which fraction(s) had the strongest ETGA signal. The Qiagen Rotor-Gene Q qPCR machine was used.

Critical values were calculated to obtain expected “limit of positivity” (LOP) values for each microorganism test run, using specimen cfu/mL and ETGA Ct values (lowest Ct value from fractions F2, F3 and F4), and the appropriate positivity threshold Ct value. Reassuringly, the positivity thresholds were lower than the lowest observed ETGA Ct values for each NSC dataset, which were 37.49 Ct units with DTT and 38.02 Ct units without DTT. It is important to note that this feasibility testing was performed using non-sterile reusable cartridges and therefore, some low level ETGA signal is expected.

With regards to microbial detection performance, the critical values shown in Table 2-1 below indicate that the filtration workflow allows detection of all species tested (PHE 2015 report Top 12) at approximately 10^3 cfu/mL or less. *Streptococcus* Group A was the worst detected microorganism with a critical value of 3988 cfu/mL, however, this species is known to be difficult to lyse using lytic enzymes. Whilst critical values provide some indication of test performance, it is important to note that critical values assume a perfectly linear relationship between cfu/mL and ETGA Ct value, which may not be the case.

Table 2-1: Filtration LOP summary table (ranked by PHE 2015 monomicrobial incidence table 2014, high to low). Critical values (cfu/input) are derived from the specimen cfu/mL and ETGA Ct values (lowest Ct value from fractions F2, F3 and F4), and the appropriate positivity threshold Ct value.

Representative species	Gram	Rank	%	Cum. %	cfu/mL	ETGA Ct	Critical value (cfu/mL)
<i>Escherichia coli</i>	GrNeg	1	30.0	30.0	7,000	28.98	14
<i>Staphylococcus epidermidis</i>	GrPos	2	17.4	47.4	1,910	35.29	306
<i>Staphylococcus aureus</i>	GrPos	3	8.9	56.2	11,000	31.88	166
<i>Streptococcus mitis</i>	GrPos	4	7.8	64.1	11,200	33.31	455
<i>Klebsiella pneumoniae</i> T1	GrNeg	5	5.6	69.7	30	37.60	24
<i>Klebsiella pneumoniae</i> T2	GrNeg	5	5.6	69.7	690	30.61	4
<i>Streptococcus</i> Group A T1	GrPos	6	5.0	74.6	840	40.34	4464
<i>Streptococcus</i> Group A T2*	GrPos	6	5.0	74.6	1,000,000	29.96	3988
<i>Enterococcus faecalis</i>	GrPos	7	4.2	78.8	9,490	33.37	402
<i>Pseudomonas aeruginosa</i>	GrNeg	8	3.1	82.0	1,130	37.53	856
<i>Proteus mirabilis</i>	GrNeg	9	2.1	84.1	7,700	30.80	55
<i>Enterobacter cloacae</i>	GrNeg	10	1.6	85.7	6,680	30.37	35
<i>Candida albicans</i>	Candida	11	1.5	87.1	6,940	31.30	70
<i>Candida albicans</i> (+DTT)**	Candida	11	1.5	87.1	6,940	27.20	16
<i>Bacteroides</i> sp.	GrNeg	12	1.0	88.1	2,360	32.79	67

*cfu/mL value based on estimate TVC; ***C. albicans* (+DTT) critical value based on positivity threshold for NSC (+DTT)

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the
5 appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of which are incorporated by
10 reference in their entireties.

CLAIMS:

1. A method of separating microorganisms from non-microorganism cells in a non-microorganism cell-containing sample, the method comprising:
 - 5 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
 - b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
 - 10 c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter.
2. A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising:
 - 15 a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
 - b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
 - c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter
 - 20 d. detecting the absence or presence of microorganisms retained within or upon the filter.
3. The method according to claim 2, wherein step d comprises detecting an enzymatic activity or a nucleic acid molecule associated with the microorganism; detecting the microorganism directly by cytometry or microscopy; or detecting the microorganism following cell culture.
4. The method according to claim 2 or 3 wherein step d comprises steps of:
 - 30 i. lysis of the microorganisms retained within the filter following step c
 - ii. incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
 - iii. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying

enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

5. A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising:
- selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
 - filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter
 - filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter
 - lysis of the microorganisms retained within the filter following step c
 - incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
 - specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.
6. The method of any preceding claim wherein selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample comprises adding a combination of a detergent and one or more enzymes to the sample; wherein the one or more enzymes comprise a proteinase and/or a DNase, optionally wherein the proteinase is proteinase K.
7. The method of any one of claims 2 to 6 wherein following step c and prior to step d the captured microorganisms are exposed to high pH conditions, optionally wherein the pH is subsequently reduced before step d is performed, optionally wherein the pH is reduced by adding a neutralisation buffer.
8. The method of any one of claims 4 to 7 wherein step d comprises adding a lysis reagent containing the substrate nucleic acid molecule.
9. The method of any preceding claim wherein the sample comprises blood.

10. The method of any preceding claim wherein steps b and c are performed using a single filter comprising a pore size on the inlet side of the filter that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter that retains microorganisms within the filter.
- 5 11. The method of claim 10 wherein the filter comprises an asymmetric pore structure, optionally wherein the pores decrease in size between the inlet and outlet sides of the filter.
- 10 12. The method of any preceding claim wherein steps b and c are performed using a filter comprising a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter.
- 15 13. The method of any preceding claim wherein the (first and/or second) filter is a membrane.
14. The method of any preceding claim wherein step b comprises use of a filter with a pore size on the inlet side of at least 1 μm , optionally wherein the pore size on the inlet side is between 1 μm and 5 μm , such as between 1 μm and 2 μm .
- 20 15. The method of any preceding claim wherein step c comprises use of a filter with a pore size on the outlet side of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than 0.2 μm .
- 25 16. The method of any one of claims 2 to 15 wherein the step of incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms is performed at a temperature of at least around 30°C, optionally between around 30°C and 40°C or between around 32°C and 37°C, such as around 37°C.
- 30 17. The method of any one of claims 2 to 16 wherein step d is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.
- 35

18. The method of any one of claims 2 to 17 wherein all of steps a to d are performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.
19. The method according to any one of claims 4 to 18 wherein the nucleic acid modifying enzyme comprises a DNA or RNA polymerase, optionally wherein the DNA polymerase is DNA polymerase I.
20. The method according to any one of claims 4 to 19 wherein the nucleic acid modifying enzyme comprises a ligase, optionally wherein the nucleic acid modifying enzyme is an NAD-dependent ligase.
21. A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising:
- a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
 - b. separation of the intact microorganisms from the lysed cell material
 - c. lysis of the microorganisms
 - d. incubating the lysate from step c with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and
 - e. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism;
- characterised in that step c is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.
22. The method of claim 21 further characterised in that step d is performed at a temperature of at least around 30°C, optionally between around 30°C and 40°C or between around 32°C and 37°C, such as around 37°C.

23. The method of claim 21 or 22 wherein each of steps a to c is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.
- 5 24. A method of detecting the absence or presence of a microorganism infection in a subject comprising performing the method of any one of claims 2 to 23 on a sample from the subject, optionally wherein the sample comprises blood from the subject.
- 10 25. A device, composition of matter or kit for detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising:
- 15 a. a first reservoir for a first lysis reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample
- b. a second reservoir for a second lysis reagent that lyses microorganisms in the sample
- c. a sample inlet for addition of the sample to enable mixing of the sample with the first lysis reagent
- 20 d. a filtration housing comprising:
- I. a first filter which captures insoluble cellular material (precipitated nuclear material)
- II. a second filter, downstream of the first filter, which captures microorganisms
- 25 III. an outlet downstream of the second filter; and
- e. a fluid channel arrangement configured to provide selective fluid communication between the filtration housing and any one or more of the first reservoir, the second reservoir and the sample inlet.
- 30 26. The device of claim 25 further comprising a filtration housing inlet upstream of the first filter wherein the fluid channel arrangement is in fluid communication with the filtration housing inlet.
- 35 27. The device of claim 25 or claim 26 further comprising a mixing chamber in selective fluid communication with the sample inlet and the first reservoir.

28. The device, composition of matter or kit of any of claims 25 to 27 comprising an asymmetric pore structure filter that comprises the first filter and the second filter, optionally wherein the pores of the asymmetric pore structure filter decrease in size between inlet and outlet sides of the filter.

29. The device, composition of matter or kit of any of claims 25 to 28 comprising a laminate structure filter having a first layer that comprises the first filter and a second layer that comprises the second filter.

30. The device, composition of matter or kit of any of claims 25 to 29 wherein the first filter and/or the second filter is a membrane.

31. The device, composition of matter or kit of any of claims 25 to 30 wherein the first filter comprises a minimum pore size on an inlet side of at least 1 μm , optionally wherein the first filter comprises a pore size on an inlet side of between 1 μm and 5 μm , such as between 1 μm and 2 μm .

32. The device, composition of matter or kit of any of claims 25 to 31 wherein the second filter comprises a maximum pore size on an outlet side of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than 0.2 μm .

33. The device, composition of matter or kit of any of claims 25 to 32 further comprising a mixing chamber in fluid communication with the first reservoir and comprising the sample inlet.

34. The device, composition of matter or kit of any of claims 25 to 33 further comprising one or more valves to control flow of fluid into the filtration housing.

35. The device, composition of matter or kit of any of claims 25 to 34 further comprising one or more syringes and/or pistons configured to transfer fluid from the first and/or second reservoirs into the filtration housing.

36. The device, composition of matter or kit of claim 35 when dependent upon claim 33 wherein the one or more syringes and/or pistons is configured to transfer fluid into the mixing chamber.

5 37. The device, composition of matter or kit of any of claims 26 to 36 further comprising a fluid bath surrounding the filtration housing.

38. The device, composition of matter or kit of any of claims 25 to 37 wherein the inlet of the filtration housing is located relative to the outlet of the filtration housing such that a
10 net direction of flow of fluid through the device or kit is parallel to the plane of the first filter.

39. The device, composition of matter or kit of claim 38 wherein the second filter is in the same plane as the first filter and laterally offset within that plane.

15 40. The device, composition of matter or kit of claim 38 wherein the second filter is in a parallel plane to the first filter.

41. The device, composition of matter or kit of any of claims 25 to 40 further comprising a plinth configured to support the device or kit on a support plane in an orientation of use,
20 and wherein the filtration housing projects in a direction perpendicular to the support plane.

42. The device, composition of matter or kit of claim 41 wherein the inlet of the filtration housing is located towards a lower edge of the filtration housing that is closer to the plinth and the outlet of the filtration housing is towards a higher edge of the filtration housing that
25 is further from the plinth.

43. A multi-channel device, composition of matter or kit comprising a plurality of the device, composition of matter or kit of any of claims 25 to 42 in parallel wherein the first and second filters of the plurality of the device or kit are located such that a net direction of flow
30 of fluid through the multi-channel device or kit is parallel to the plane or planes of the first and second filters.

44. A kit for performing the method of any one of claims 1 to 24 comprising:
(a) a reagent that selectively lyses non-microorganism cells in the sample whilst
35 retaining intact any microorganisms present in the sample

- (b) a first filter that prevents passage of insoluble cellular material through the filter
- (c) a second filter that retains microorganisms within or upon the filter

45. The kit of claim 44 further comprising

- 5 (d) detection means for detecting the absence or presence of microorganisms retained within or upon the filter

46. The kit of claim 45 wherein the detection means comprises a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms

10

47. The kit of claim 45 or 46 wherein the detection means comprise or further comprise reagents for nucleic acid amplification, optionally wherein the reagents for nucleic acid amplification comprise primers and/or probes that hybridise to a microorganism nucleic acid molecule or a nucleic acid molecule which acts as a substrate for nucleic acid

15 modifying activity of the microorganisms

48. The kit of any one of claims 44 to 47 further comprising a reagent capable of lysing microorganisms retained within or upon the filter, optionally wherein the kit comprises a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms included in the reagent capable of lysing microorganisms retained within or upon the filter.

20

49. The kit of any one of claims 44 to 48 wherein the reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample comprises a combination of a detergent and one or more enzymes; wherein the one or more enzymes optionally comprise a proteinase and/or a DNase.

25

50. The kit of any one of claims 44 to 49 further comprising:

30

- a. a high pH reagent and/or
- b. a neutralisation buffer.

51. The kit of any one of claims 44 to 50 wherein the sample comprises blood.

52. The kit of any one of claims 44 to 51 wherein the first filter comprises a pore size on the inlet side of at least 1 μm , optionally wherein the pore size on the inlet side is between 1 μm and 5 μm , such as between 1 μm and 2 μm .

5 53. The kit of any one of claims 44 to 52 wherein the second filter comprises a pore size on the outlet side of no more than 0.5 μm , no more than 0.45 μm , no more than 0.25 μm , no more than 0.22 μm or no more than 0.2 μm .

10 54. The kit of any one of claims 44 to 53 wherein the first and second filter are provided as a single filter comprising a pore size on the inlet side of the filter that prevents passage of insoluble cellular material and a pore size on the outlet side of the filter that retains microorganisms within the filter.

15 55. The kit of claim 54 wherein the filter comprises an asymmetric pore structure, optionally wherein the pores decrease in size between the inlet and outlet sides of the filter.

20 56. The kit of any one of claims 44 to 53 wherein the first and second filter are provided as a laminate structure comprising a first layer that prevents passage of insoluble cellular material and a second layer that retains microorganisms within the filter.

57. The kit of any one of claims 44 to 56 wherein the first and/or second filter is a membrane.

25 58. The kit of any one of claims 44 to 57 wherein the nucleic acid modifying enzyme comprises:

- a. a DNA or RNA polymerase, optionally wherein the DNA polymerase is DNA polymerase I; and/or
 - b. a ligase, optionally wherein the ligase is an ATP- and/or NAD-dependent
- 30 ligase.

59. The kit of any one of claims 44 to 58 further comprising a filtration housing for housing, or that houses, the first and second filter, optionally wherein the filtration housing comprises an inlet and an outlet and the inlet of the filtration housing is located relative to

35 the outlet of the filtration housing such that a net direction of flow of fluid through the

filtration housing is parallel to the plane of the first filter when housed in the filtration housing.

60. The kit of claim 59 wherein the housed second filter is in the same plane as the first
5 filter and laterally offset within that plane and/or wherein the housed second filter is in a parallel plane to the housed first filter.

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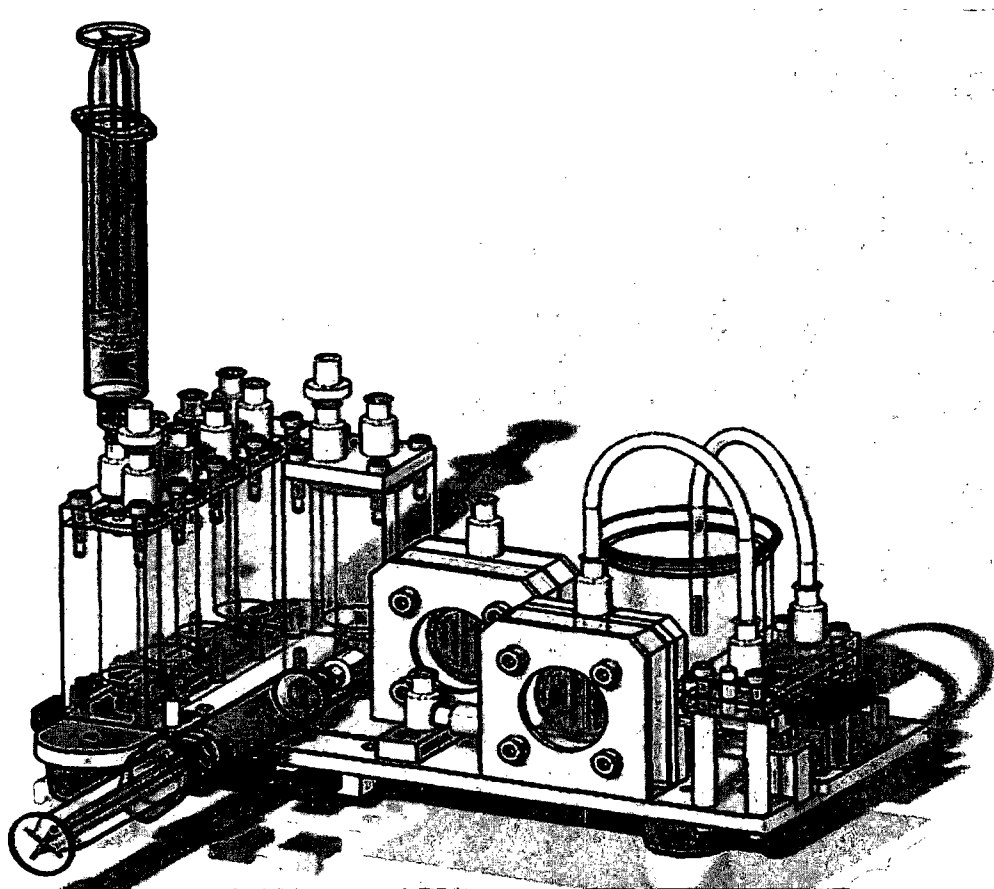


Figure 1

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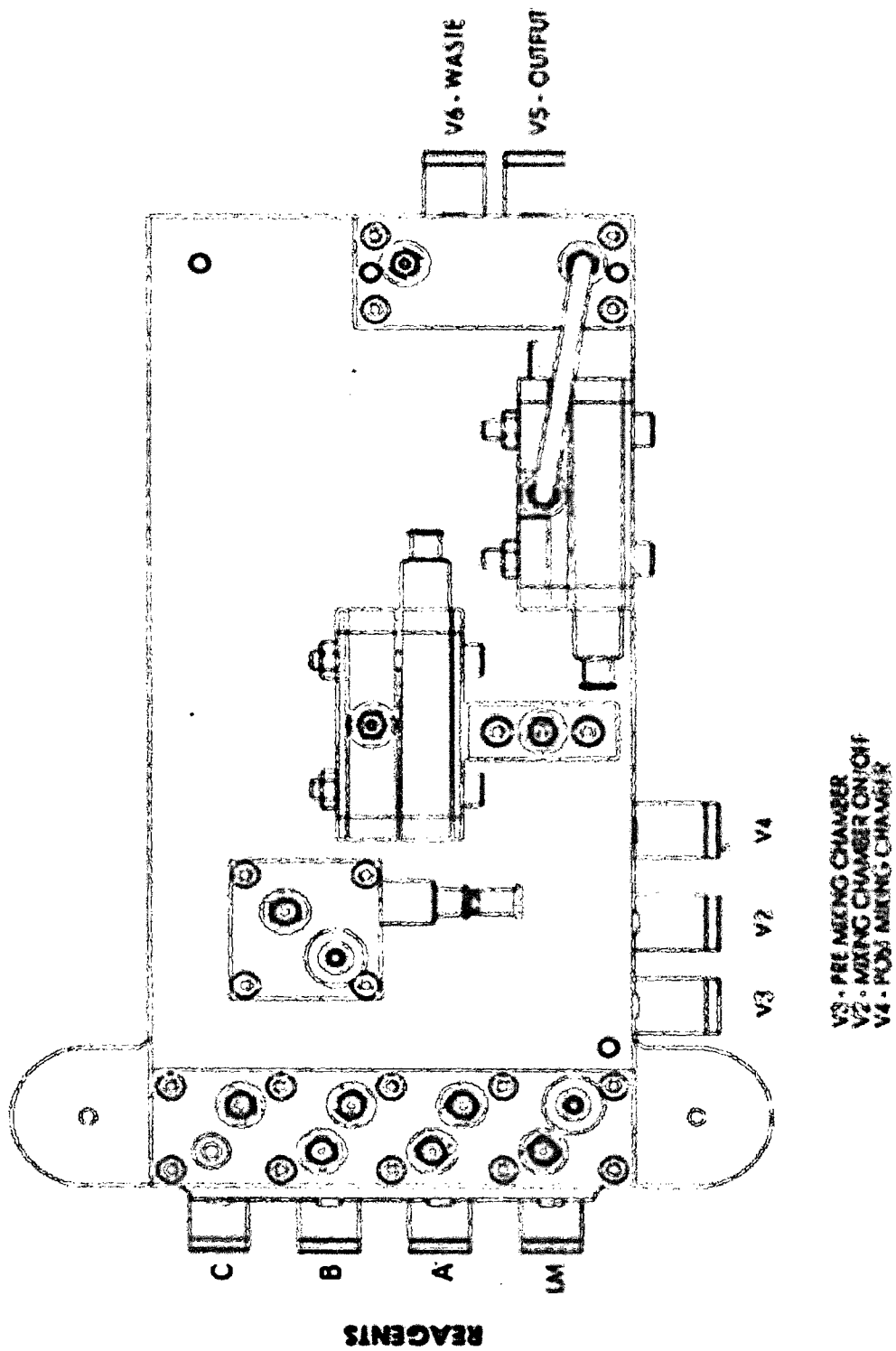


Figure 2

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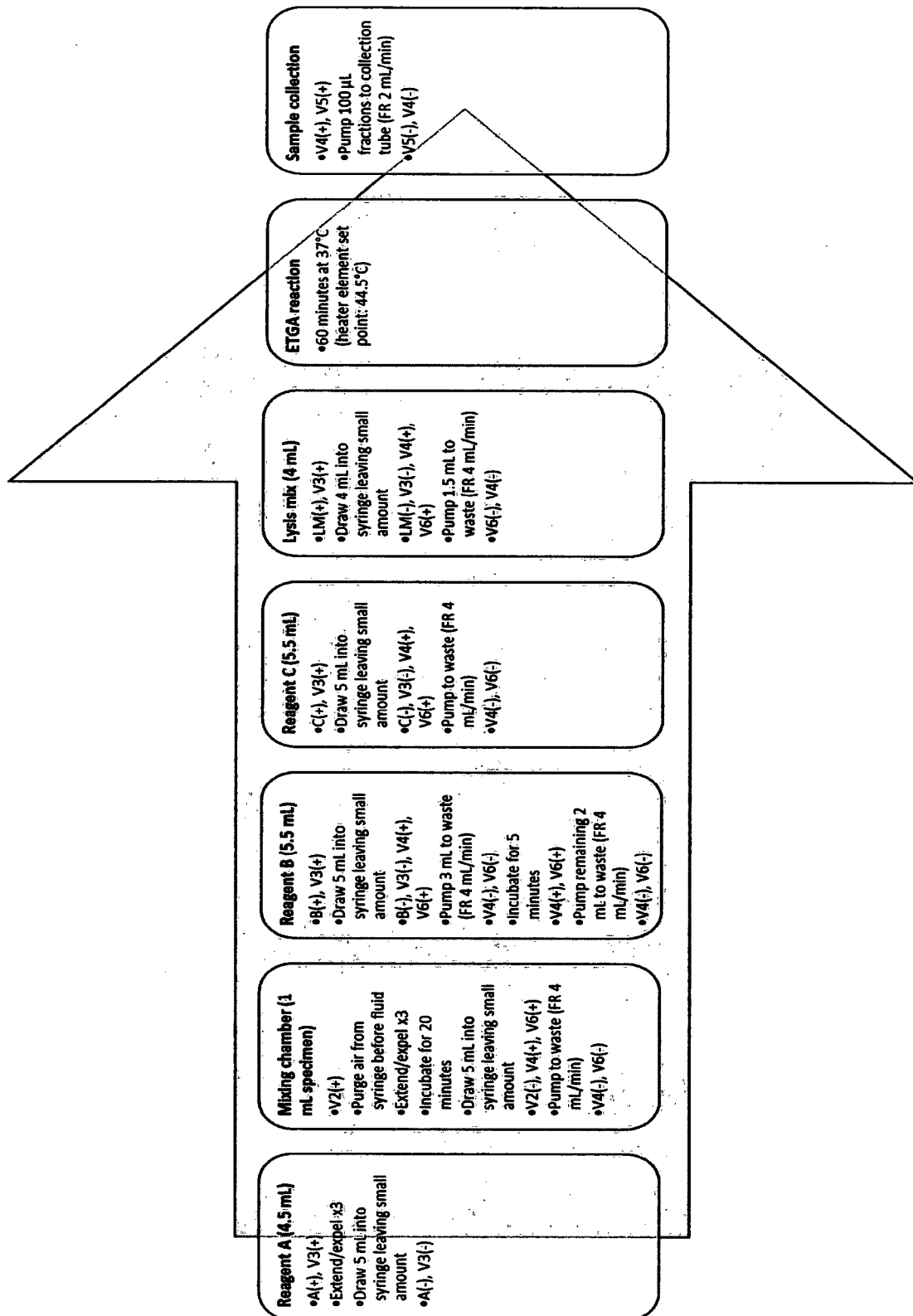


Figure 3

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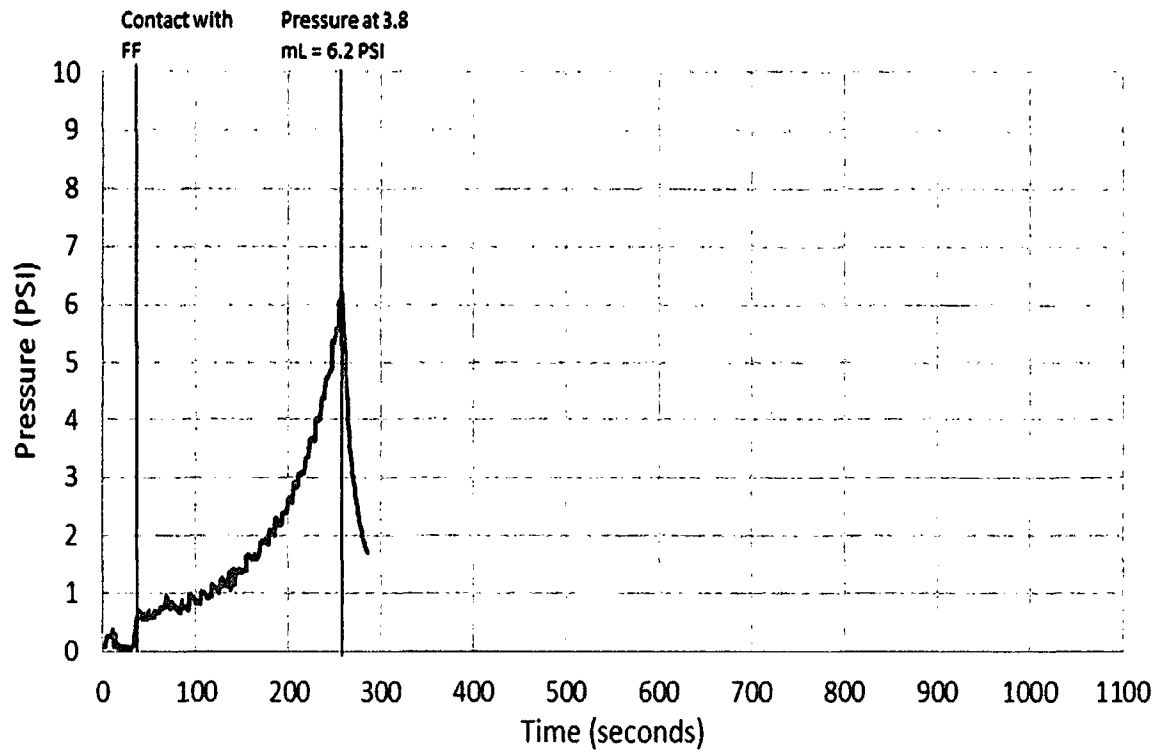


Figure 4A

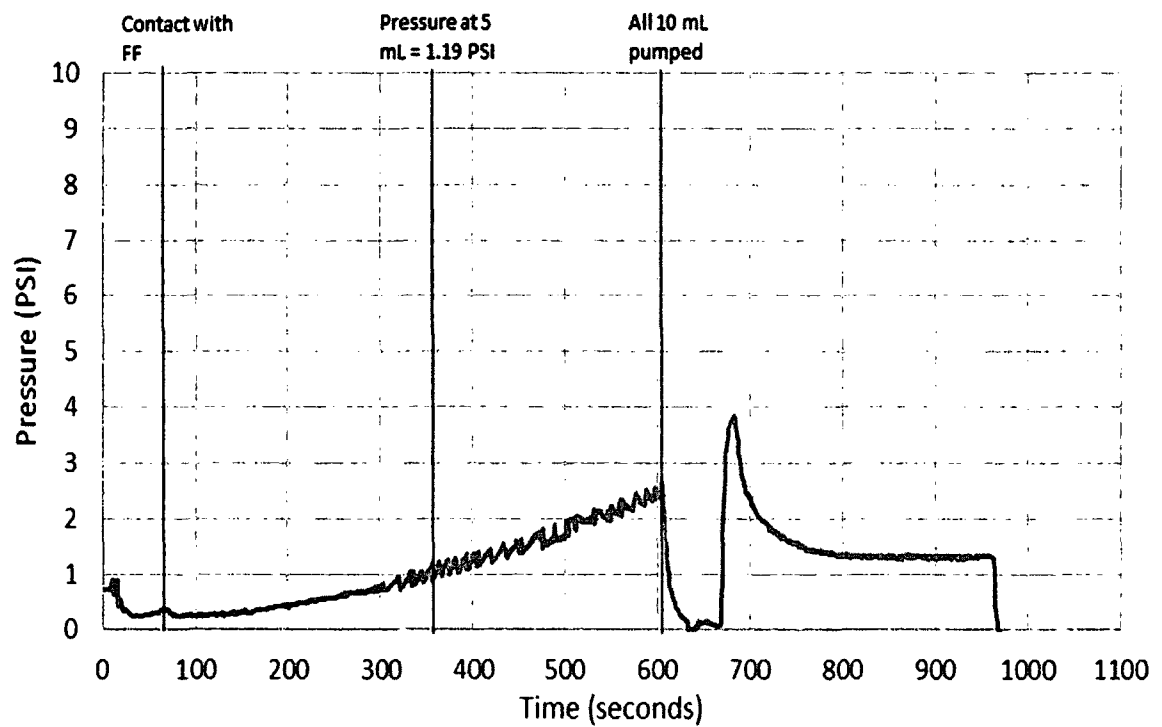


Figure 4B

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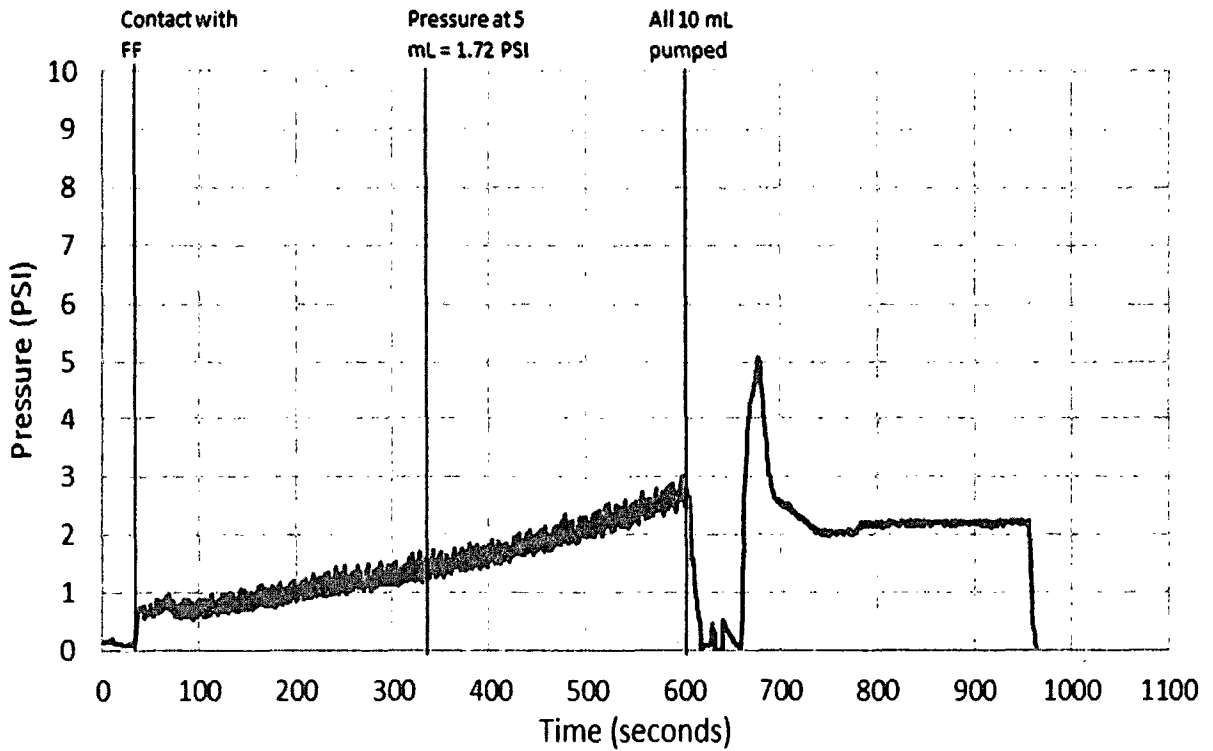


Figure 4C

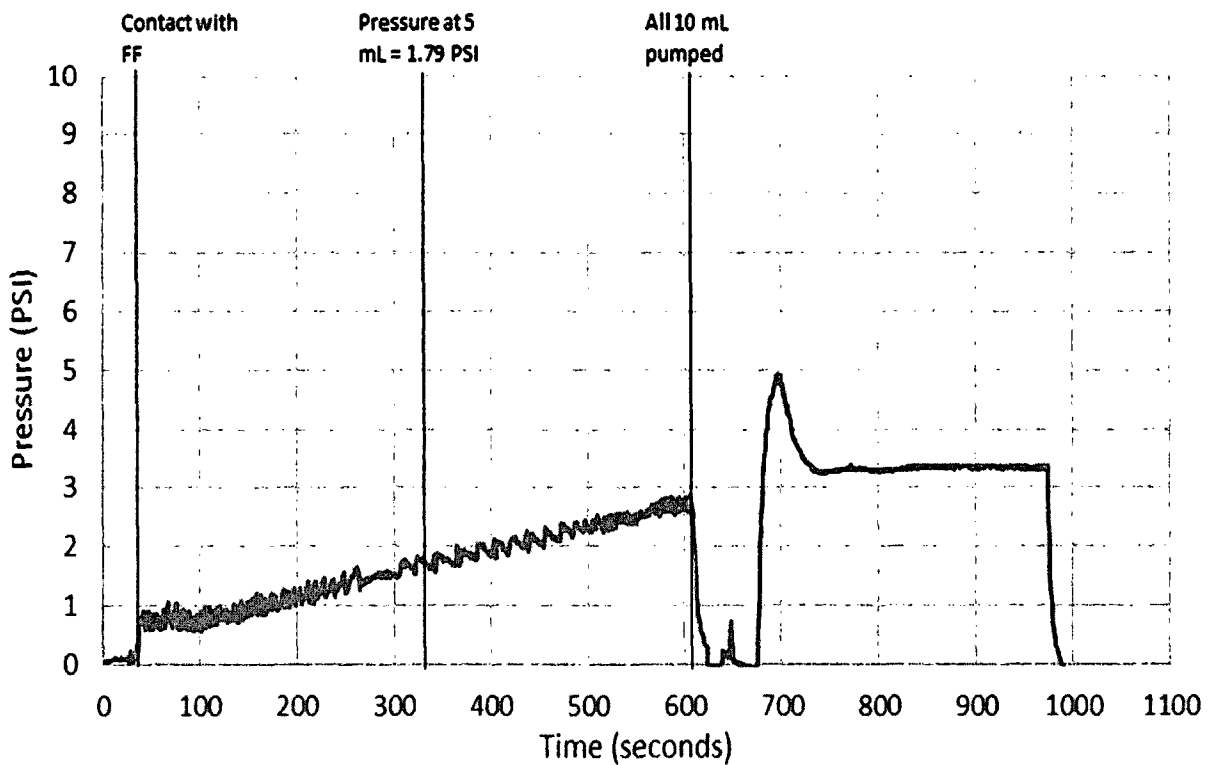


Figure 4D

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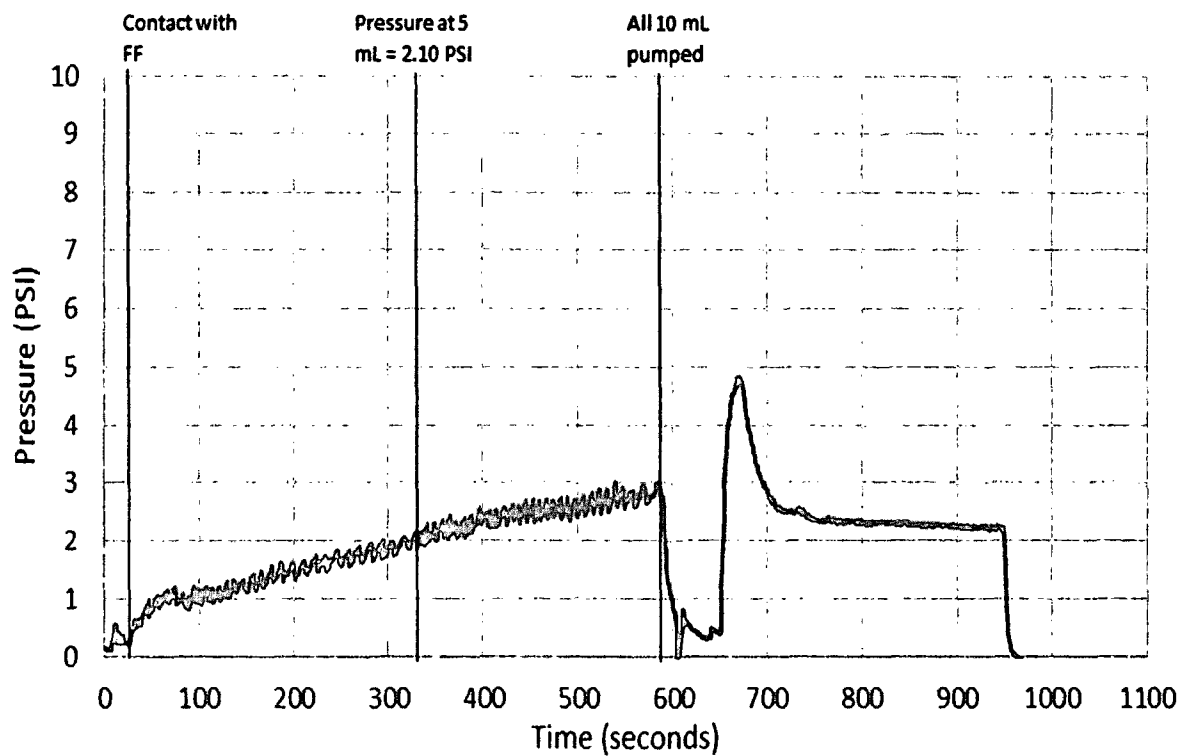


Figure 4E

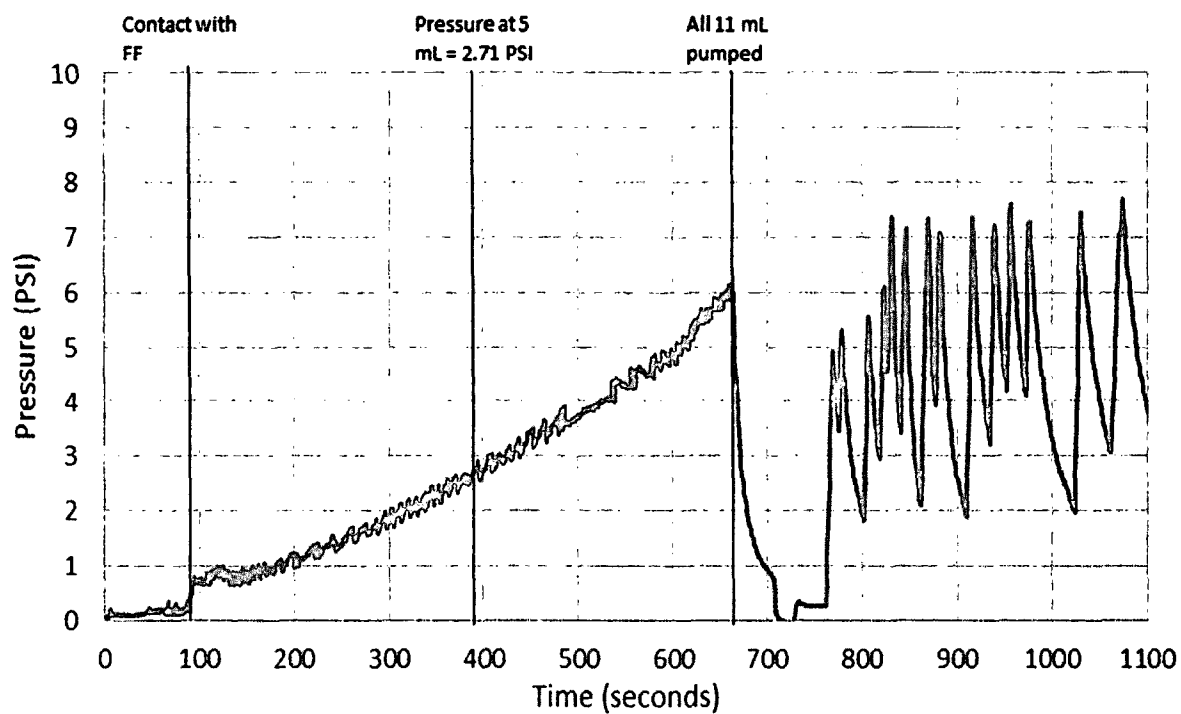


Figure 4F

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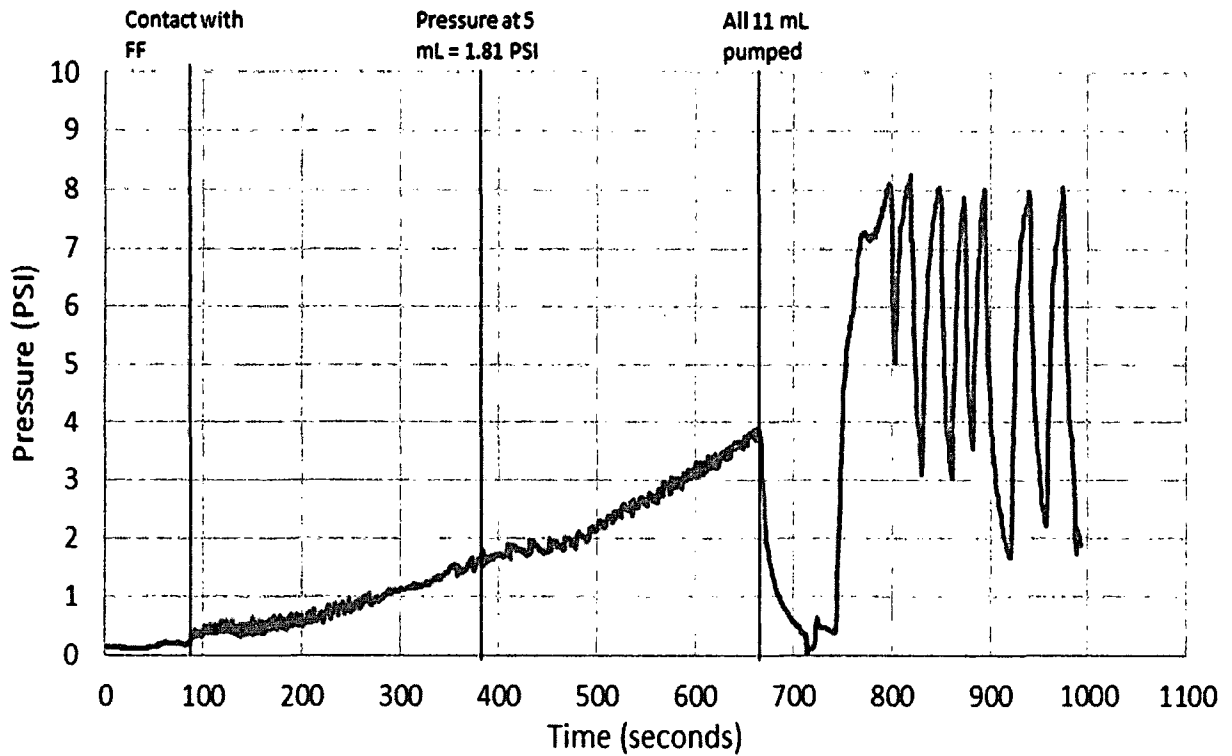


Figure 4G

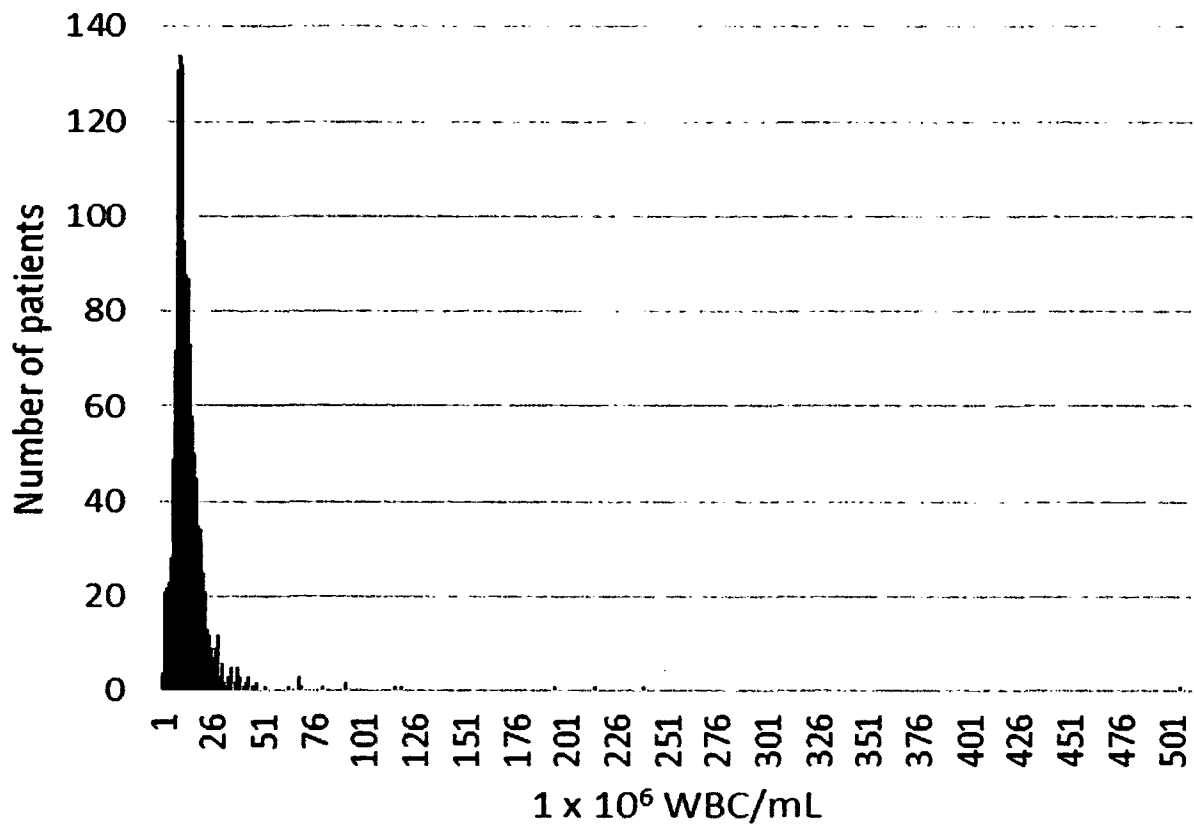


Figure 5

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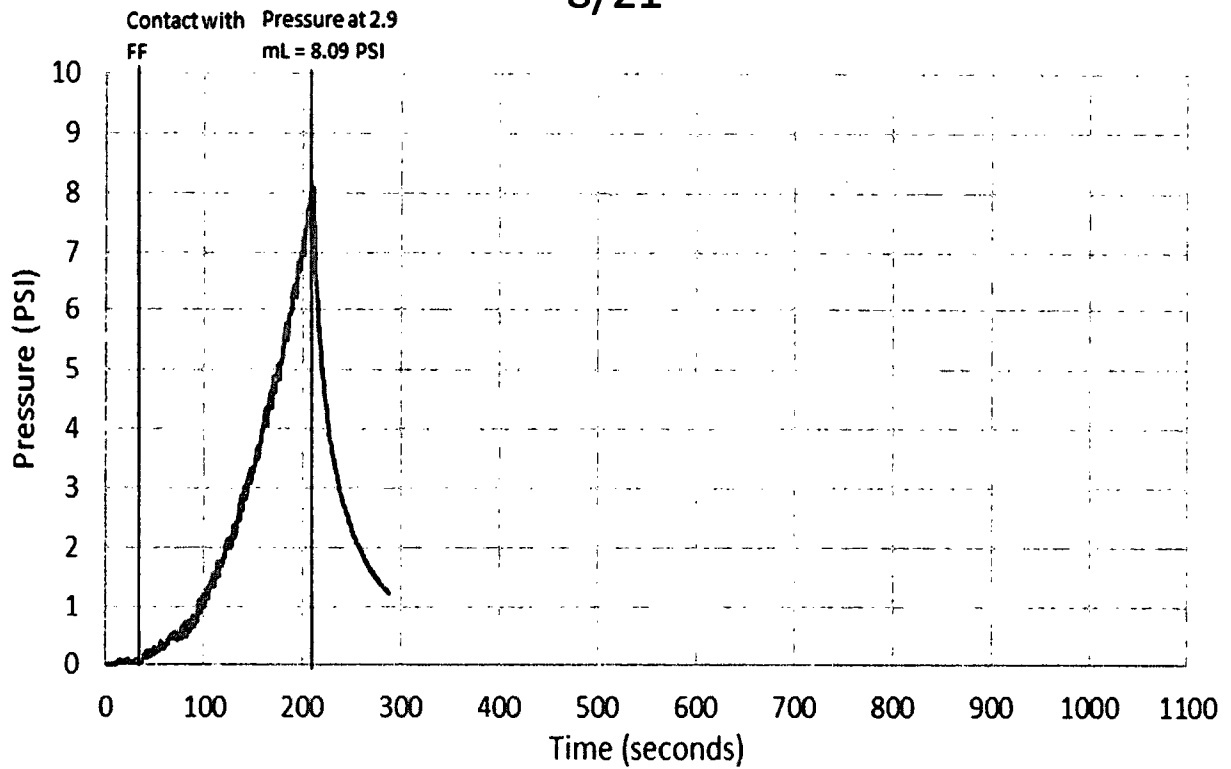


Figure 6A

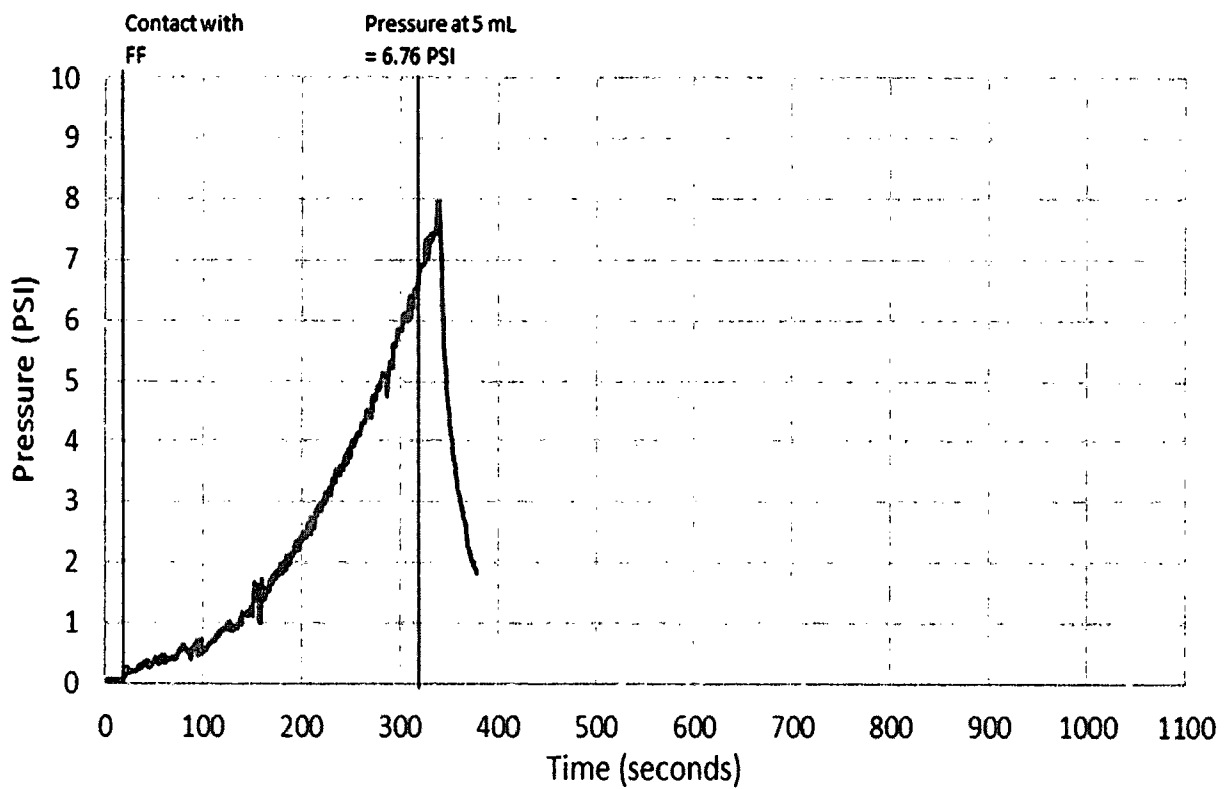


Figure 6B

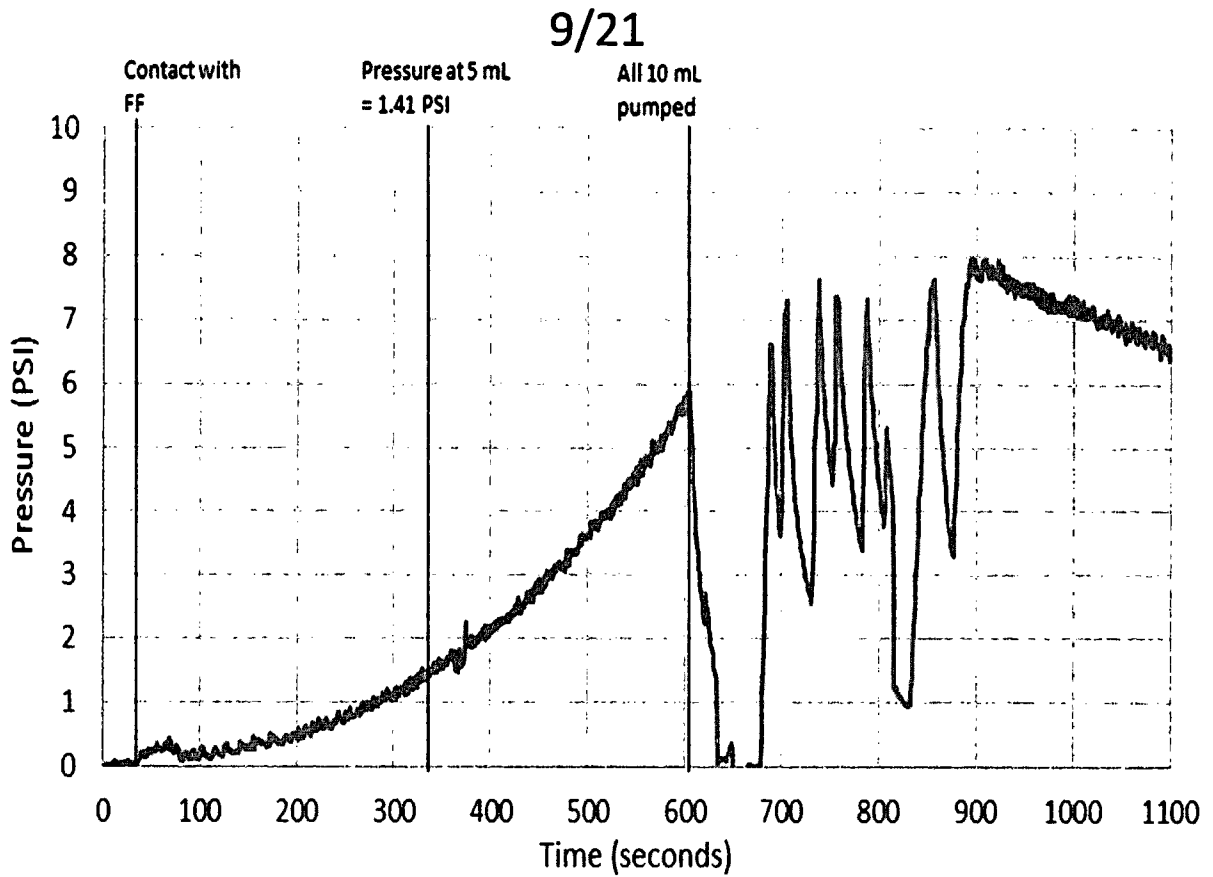


Figure 6C

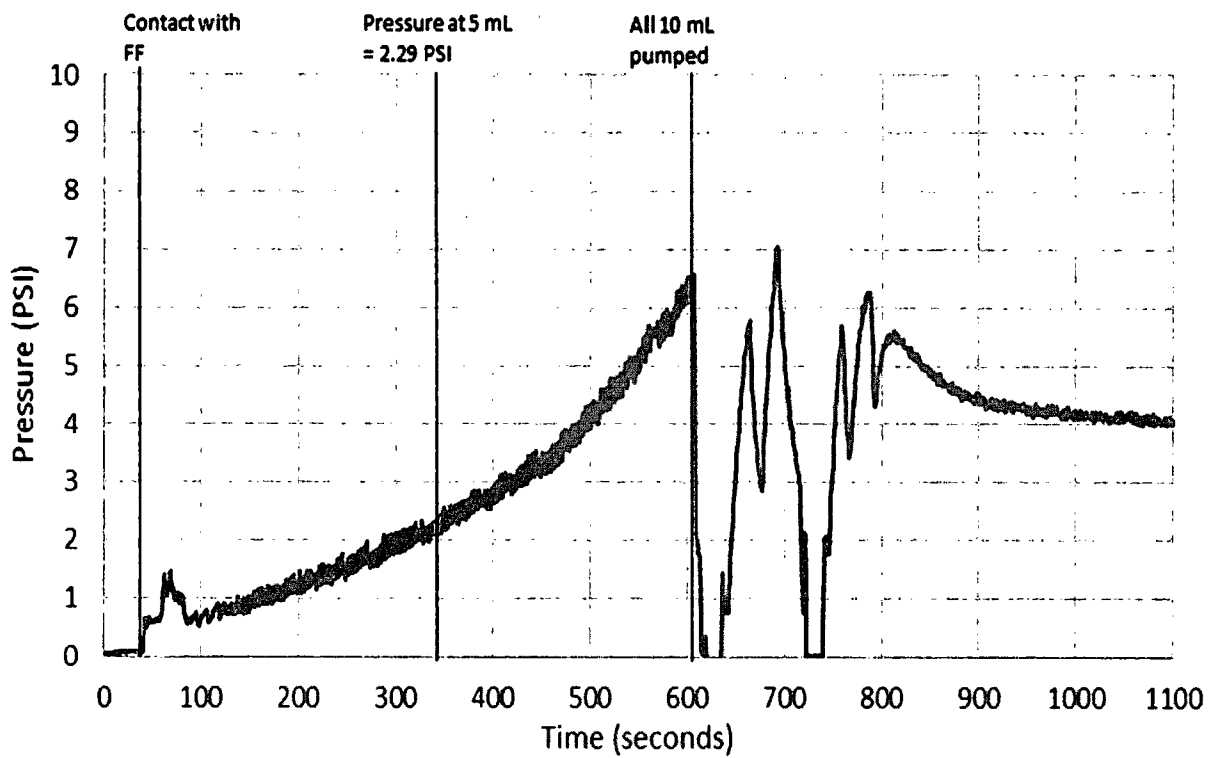


Figure 6D

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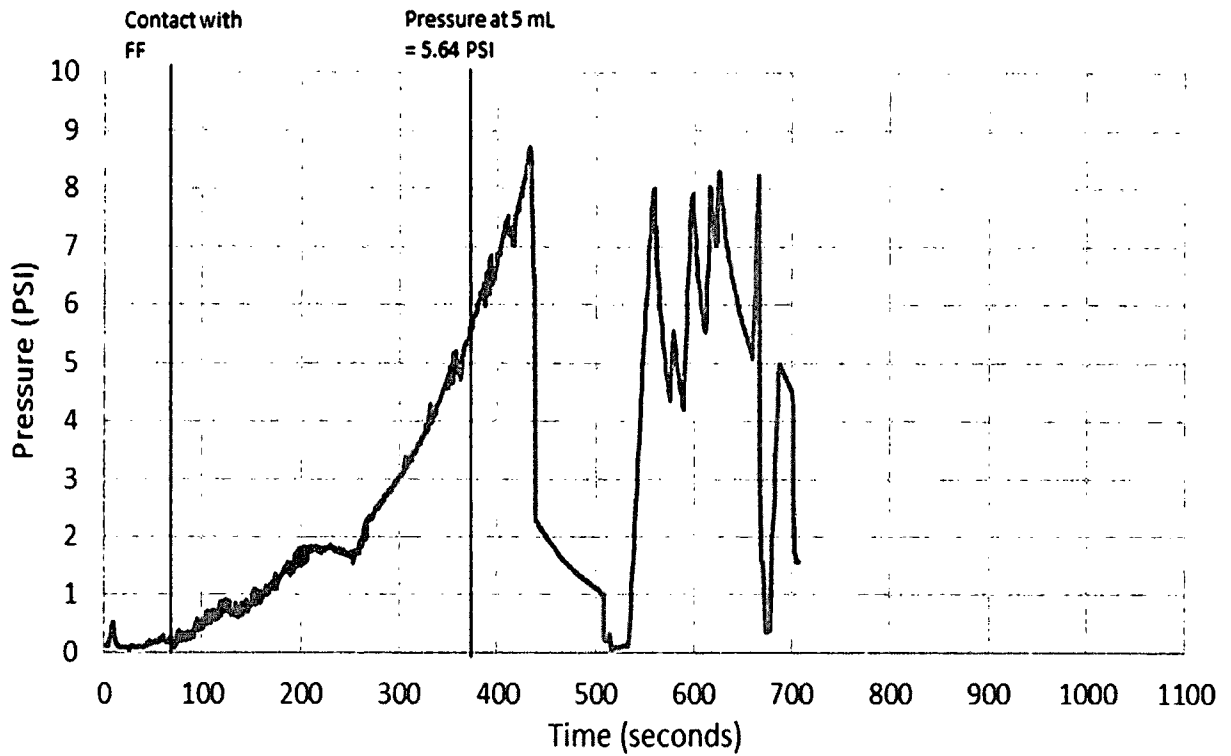


Figure 6E

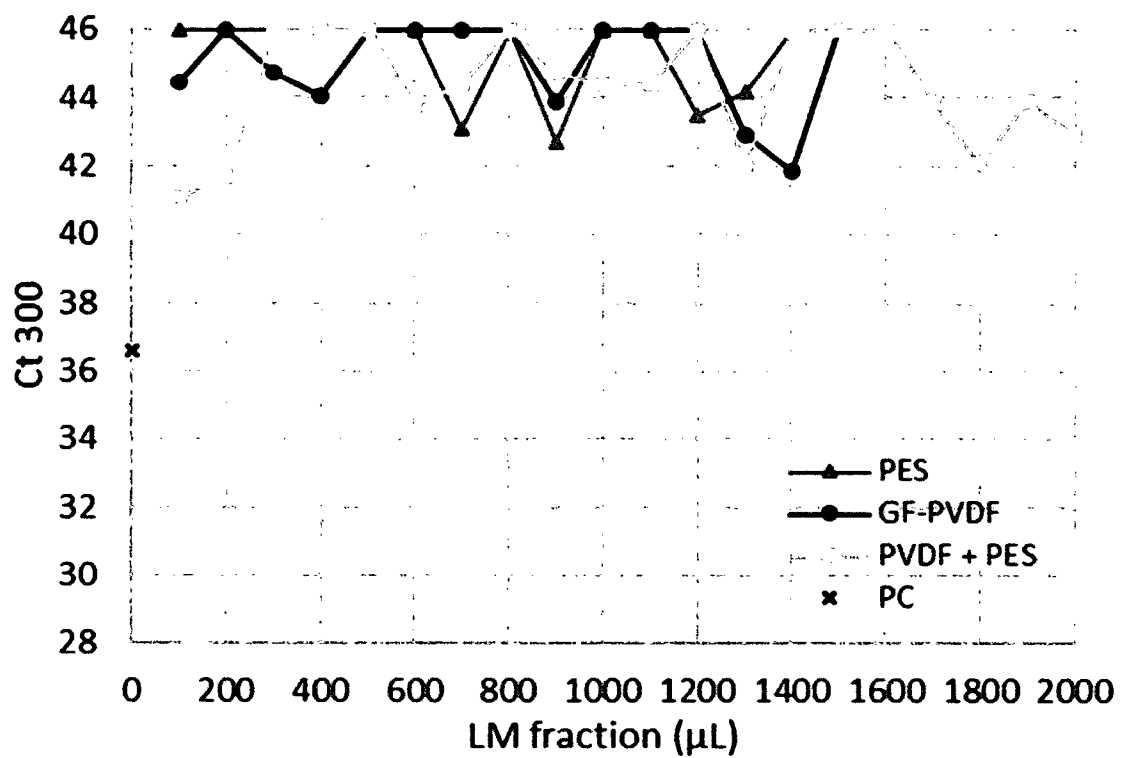


Figure 7A

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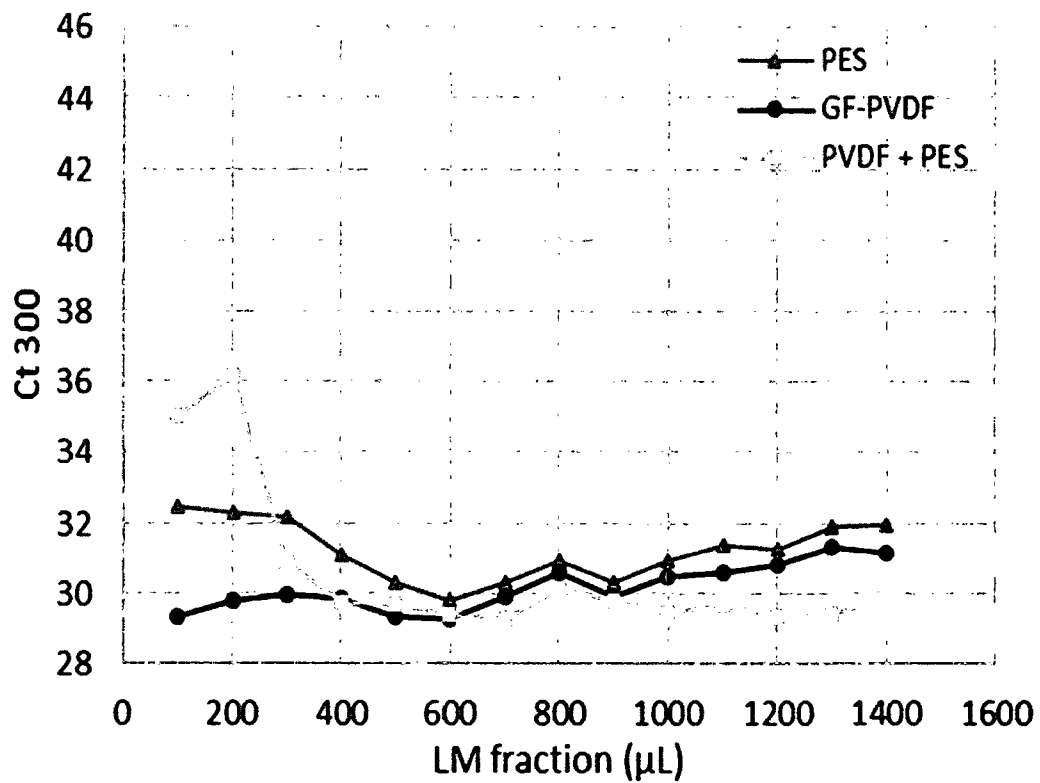


Figure 7B

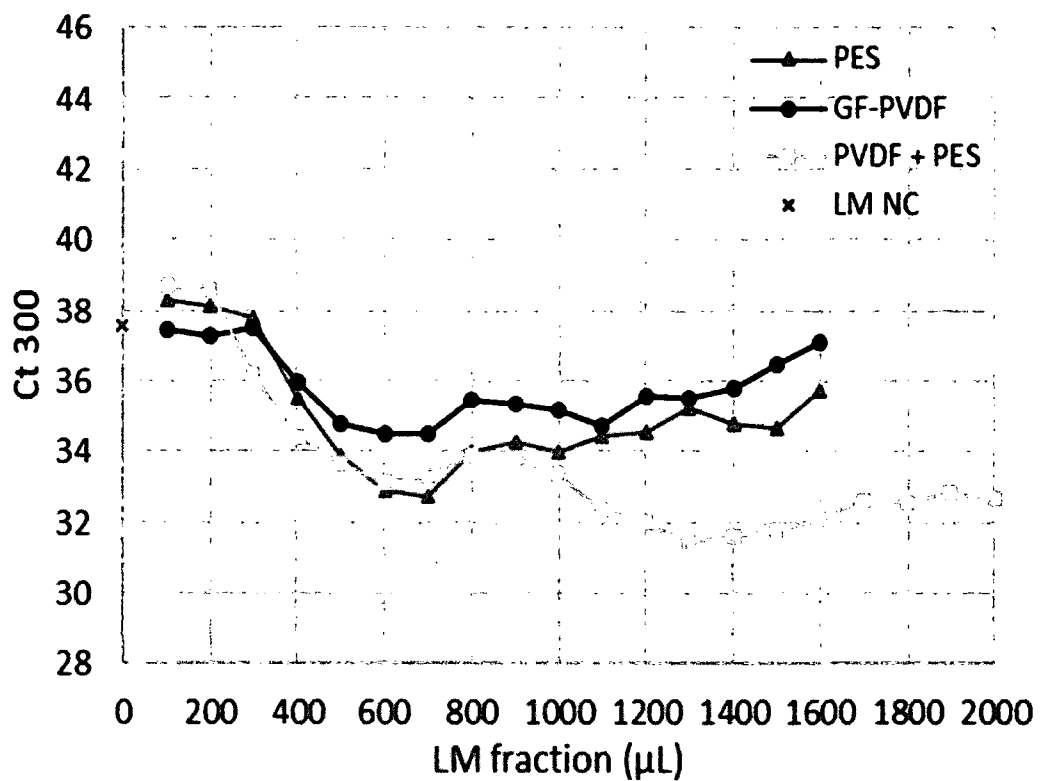


Figure 7C

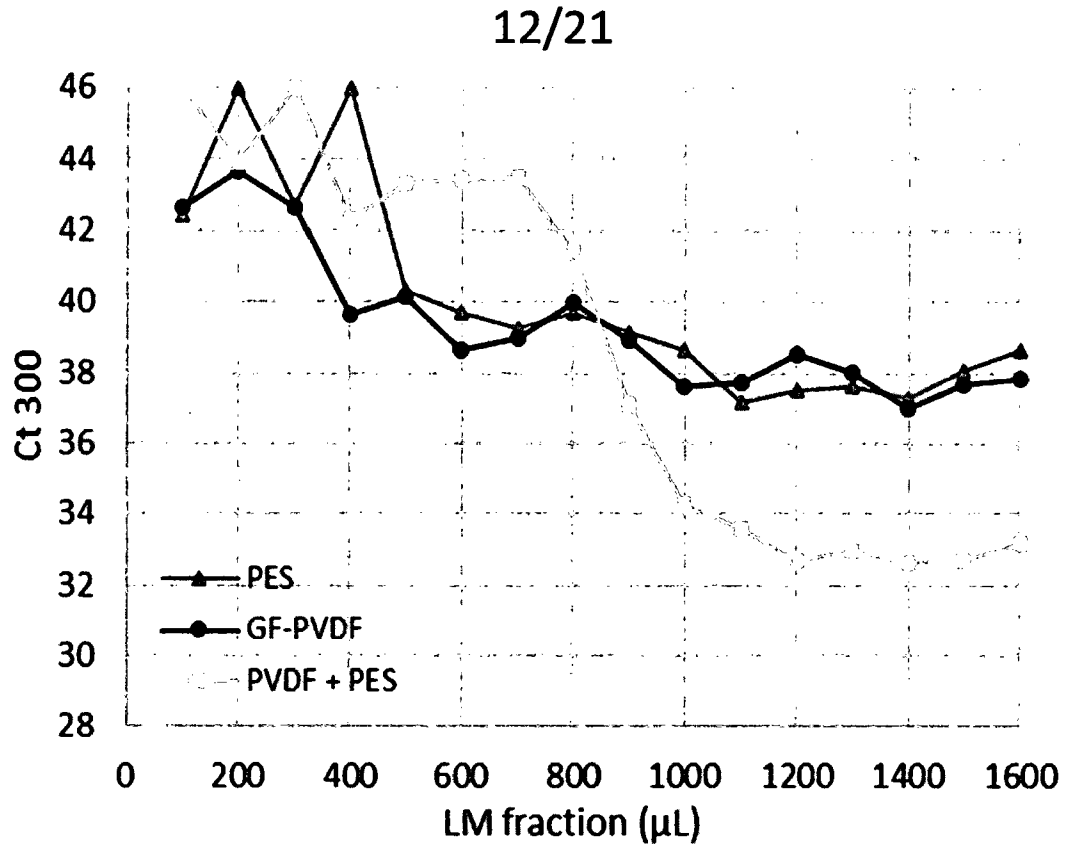


Figure 7D

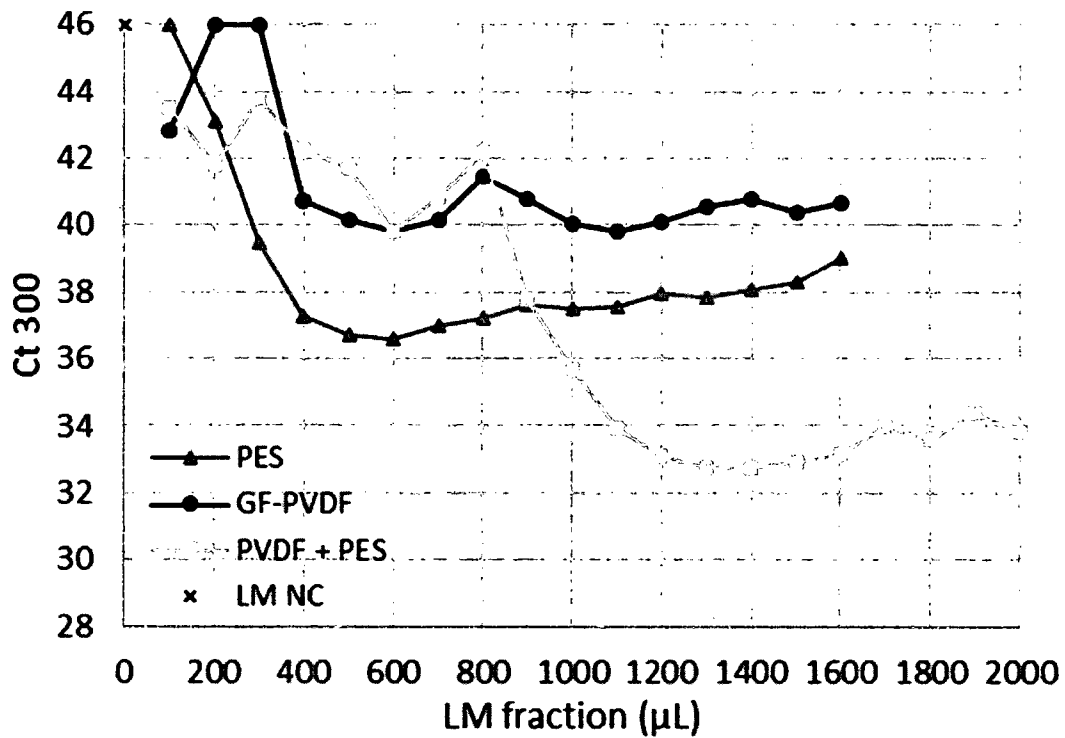


Figure 7E

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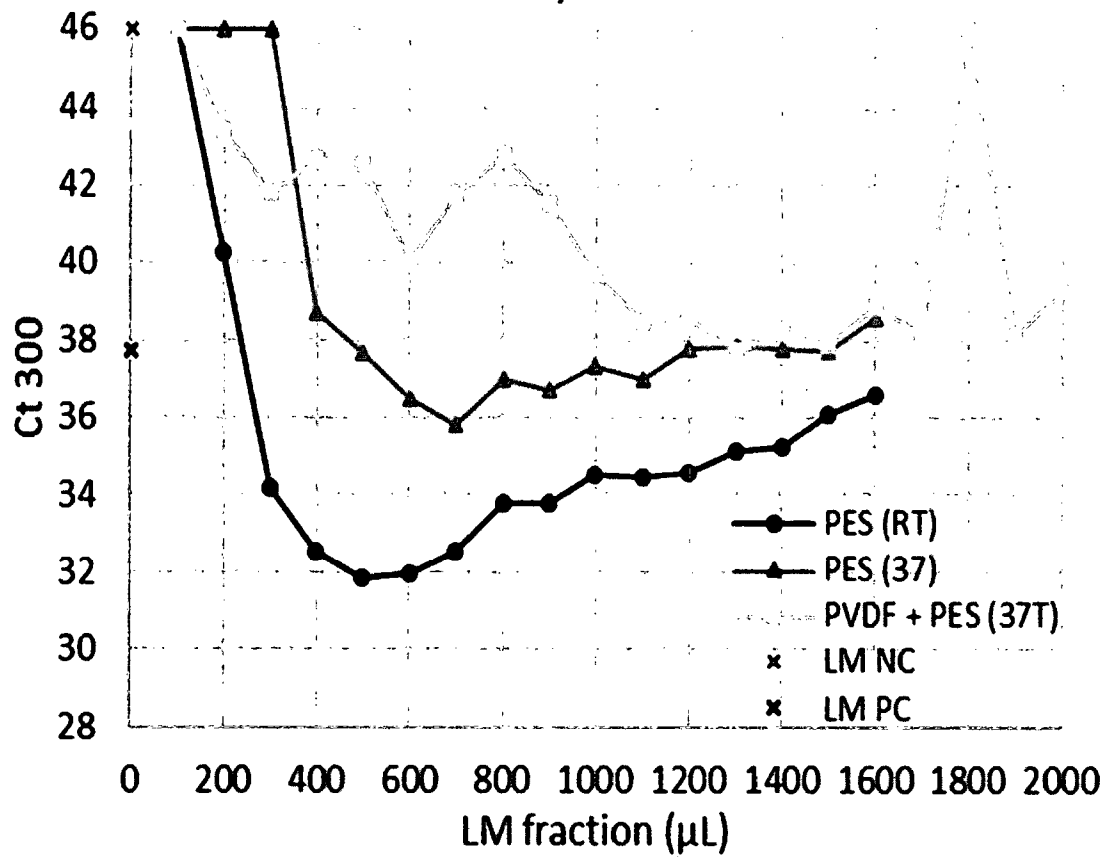


Figure 8A

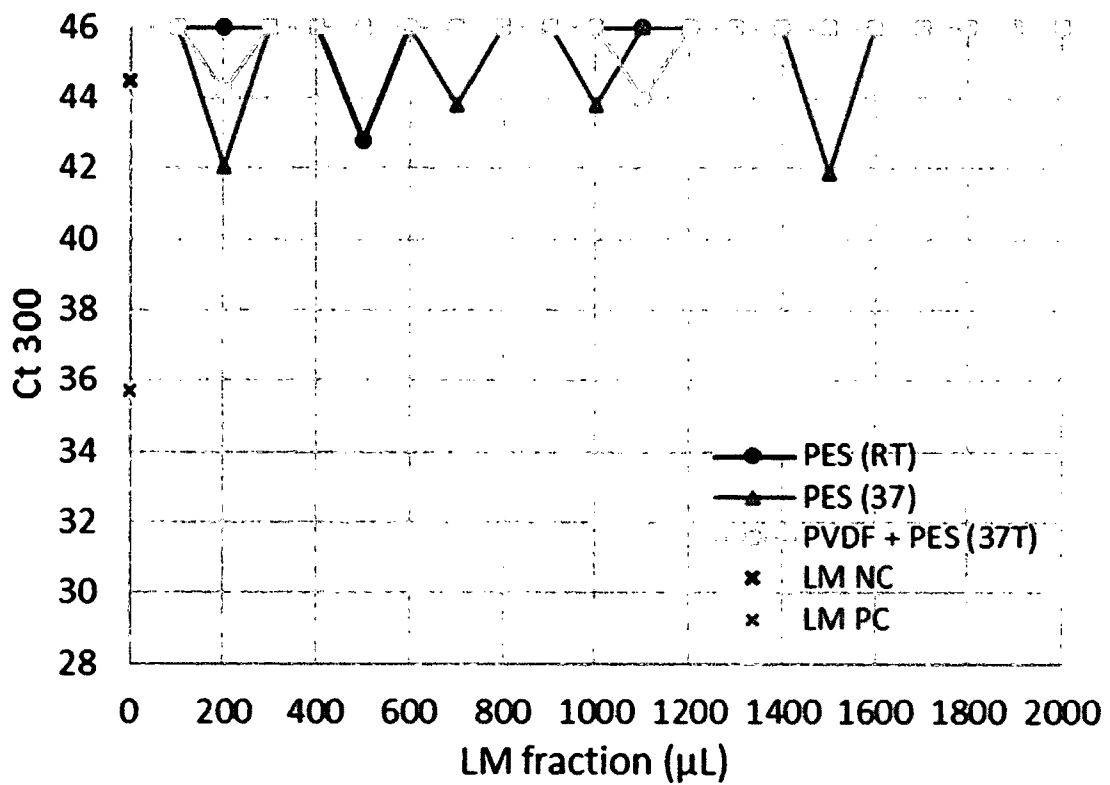


Figure 8B

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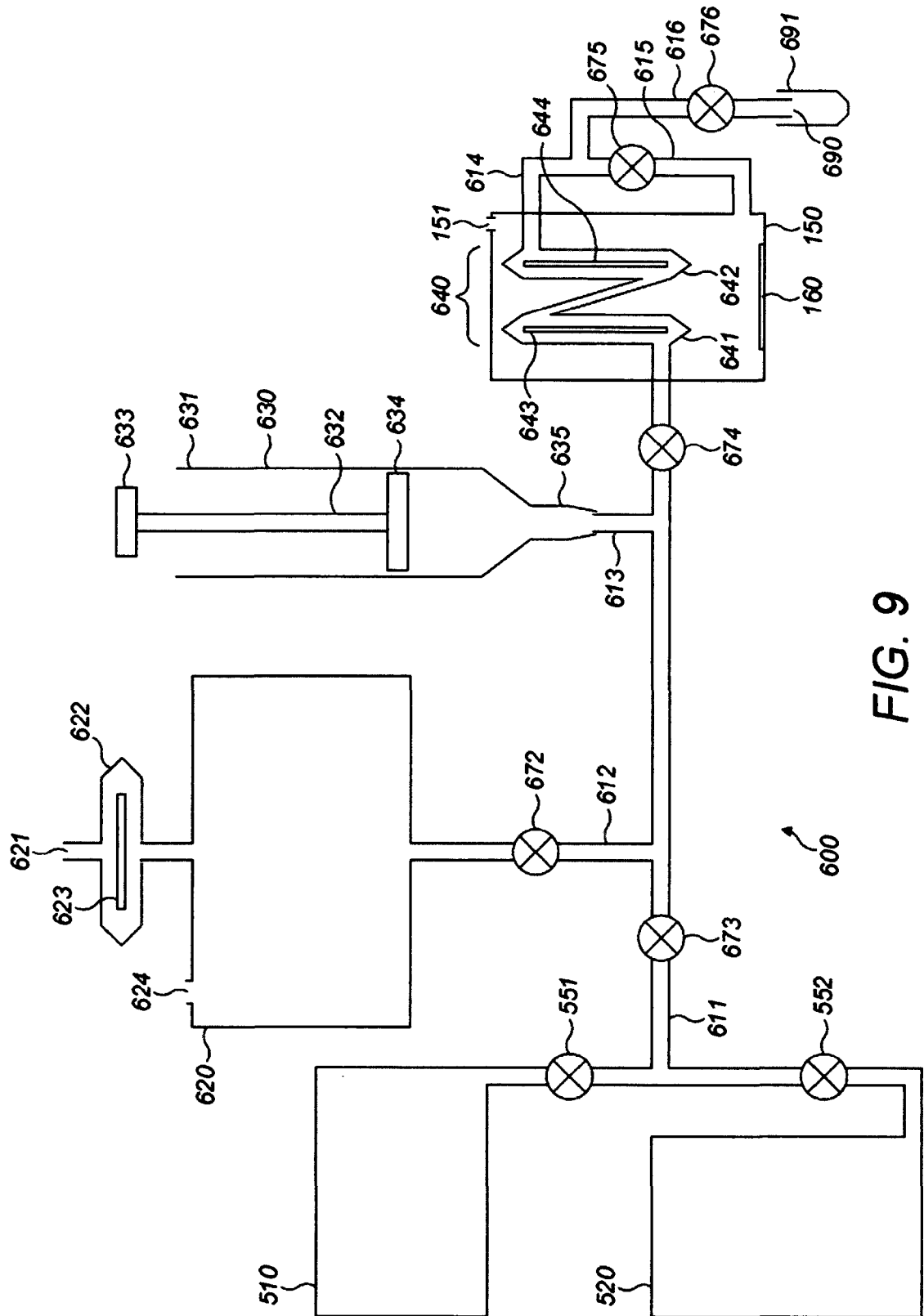


FIG. 9

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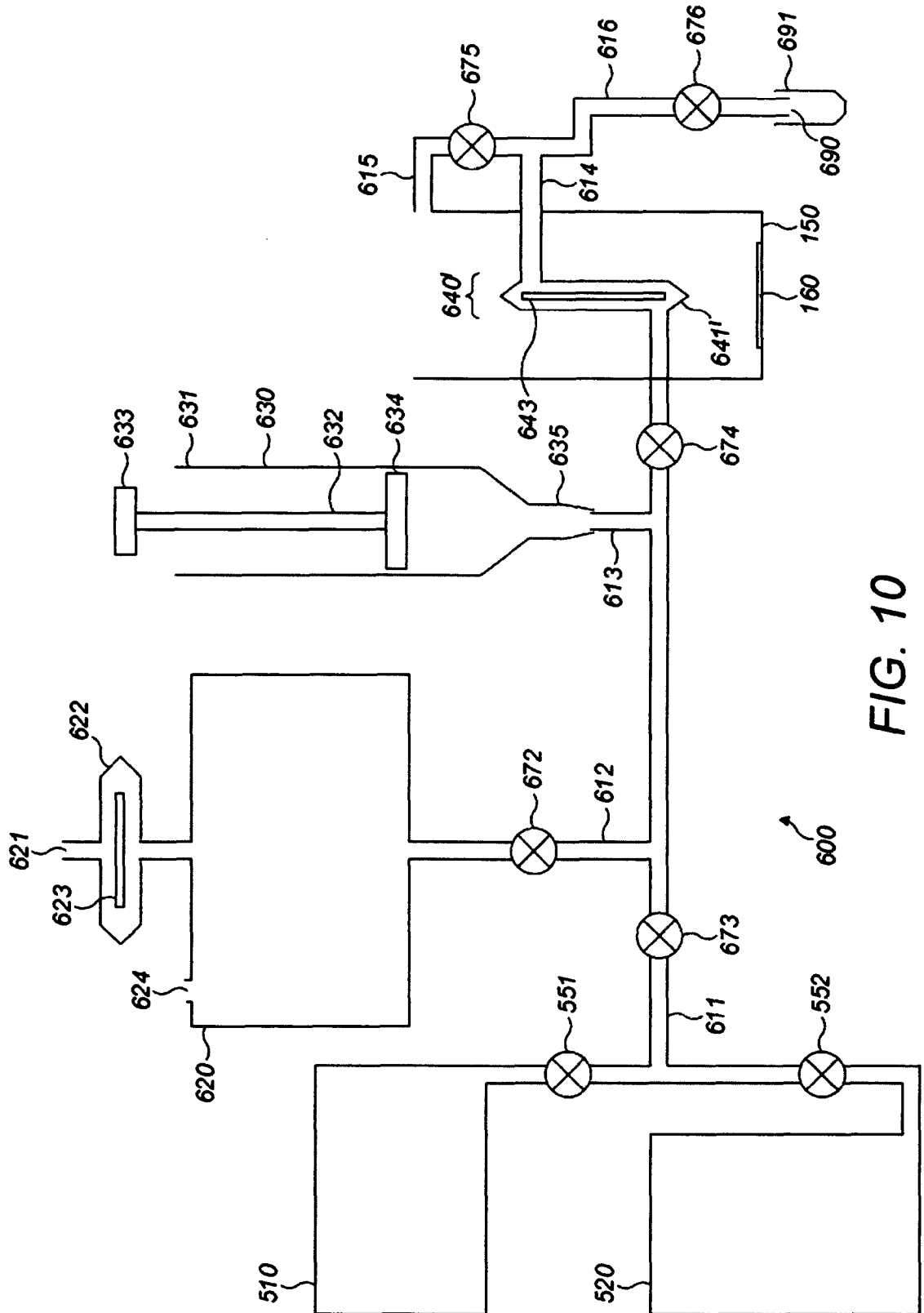


FIG. 10

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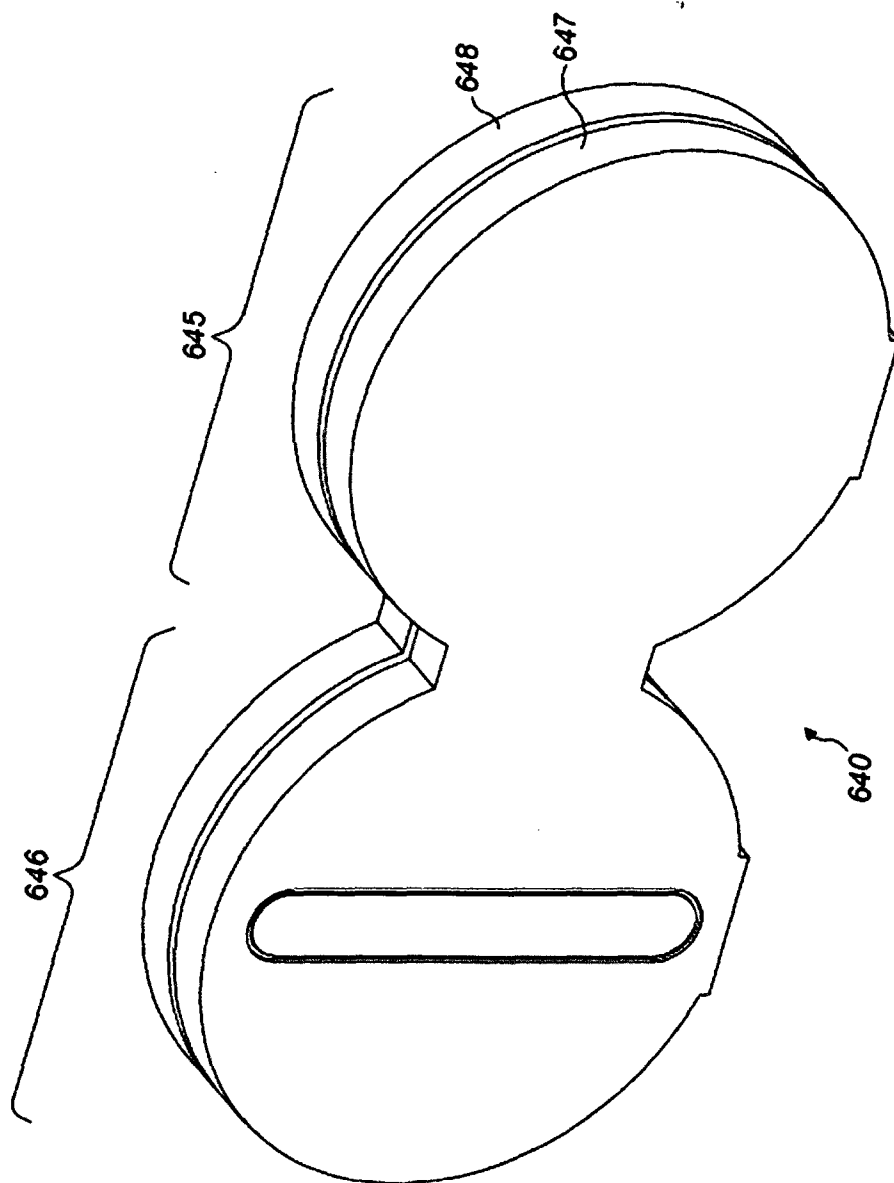


FIG. 11

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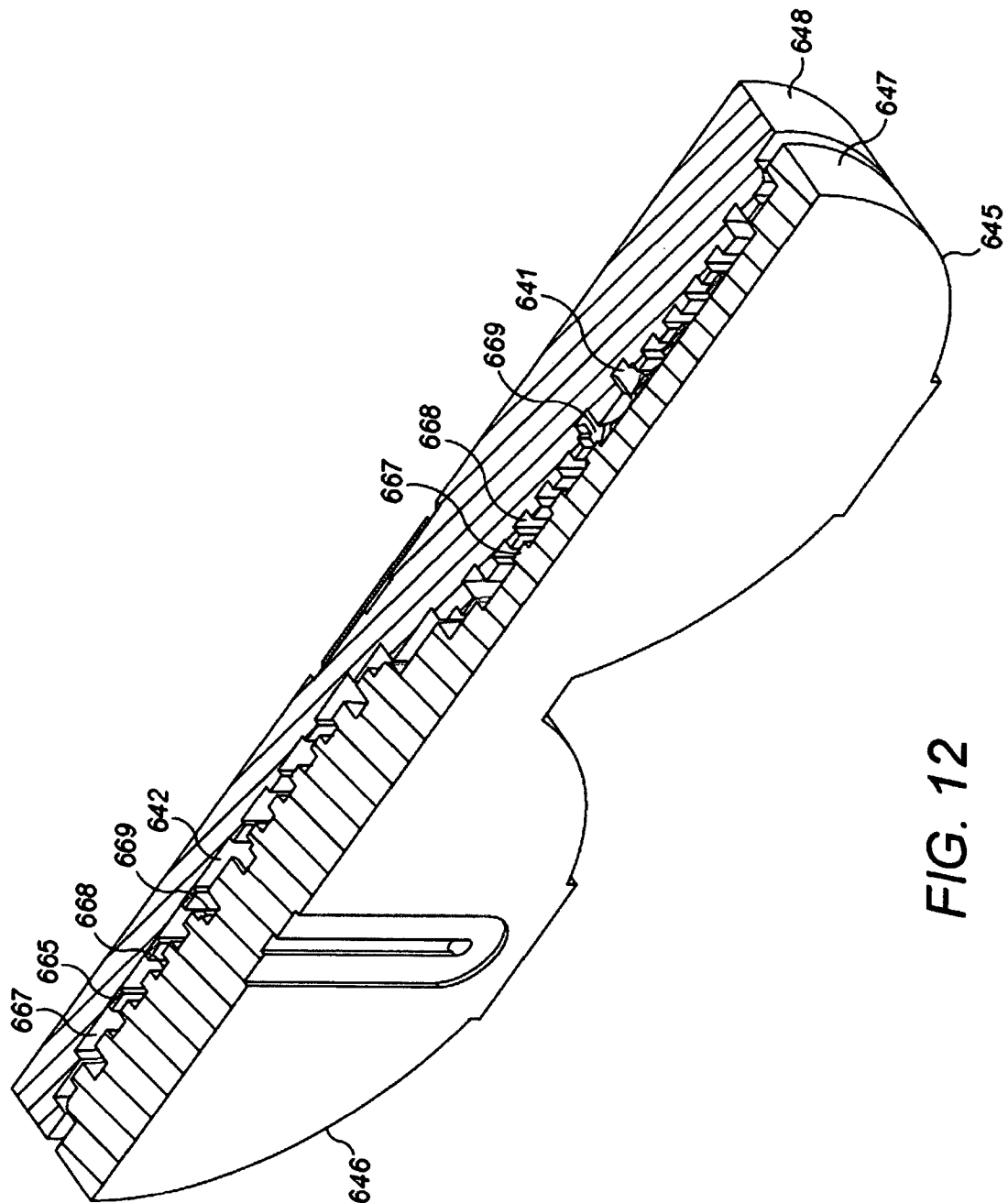


FIG. 12

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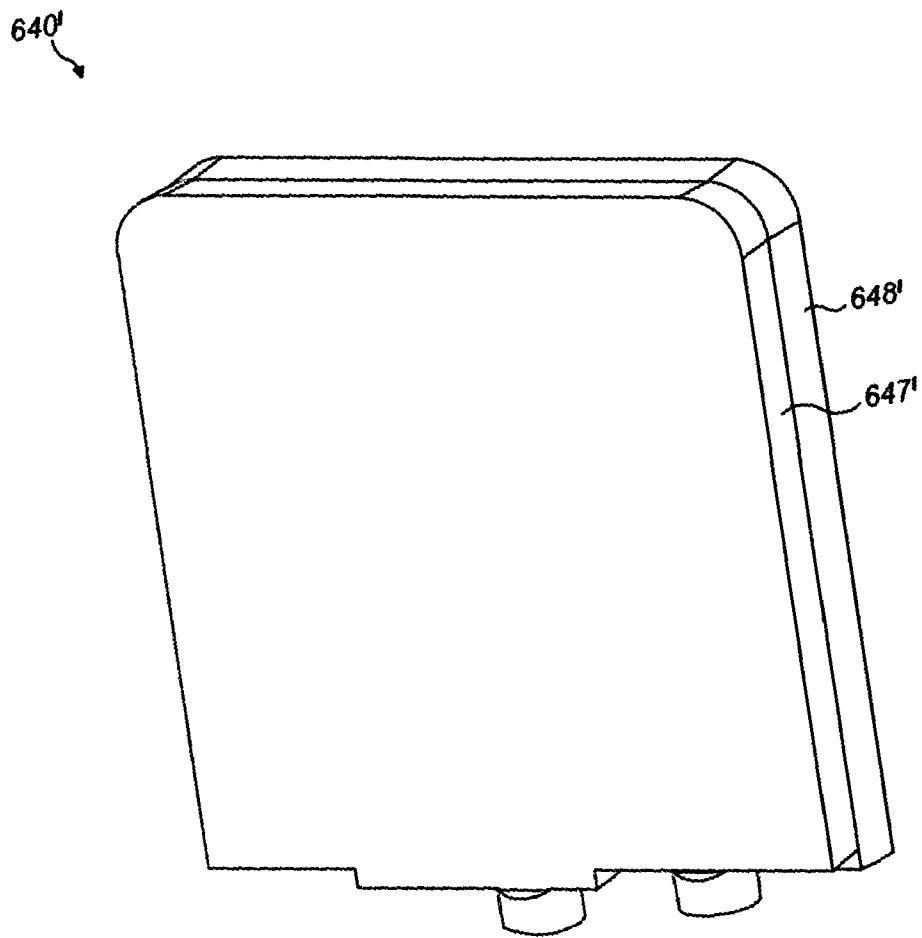


FIG. 13

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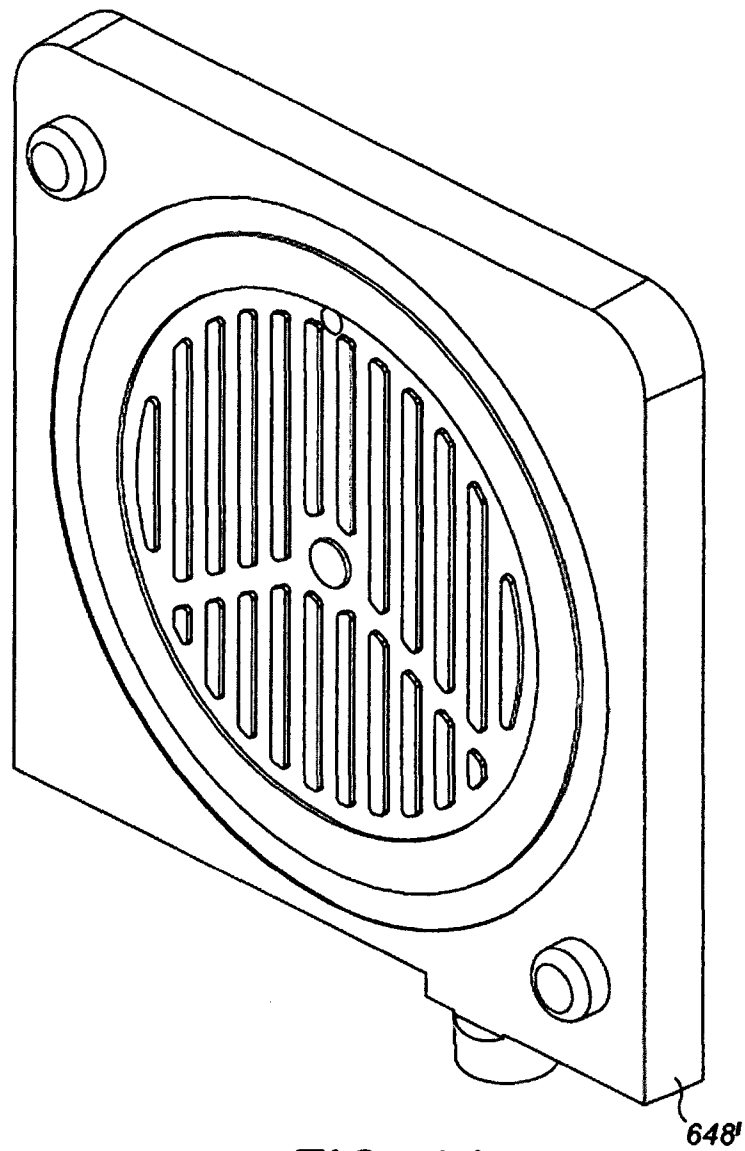
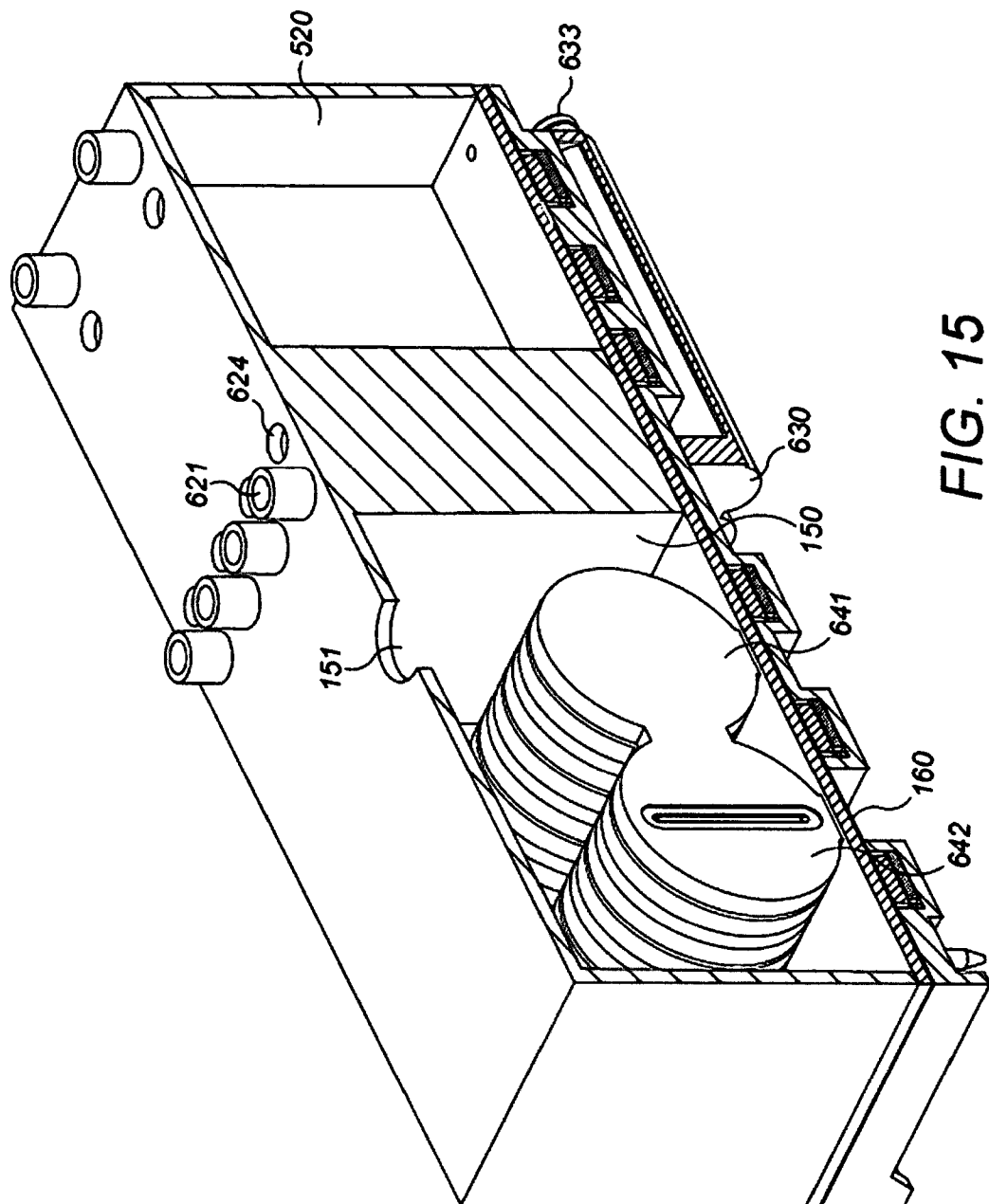
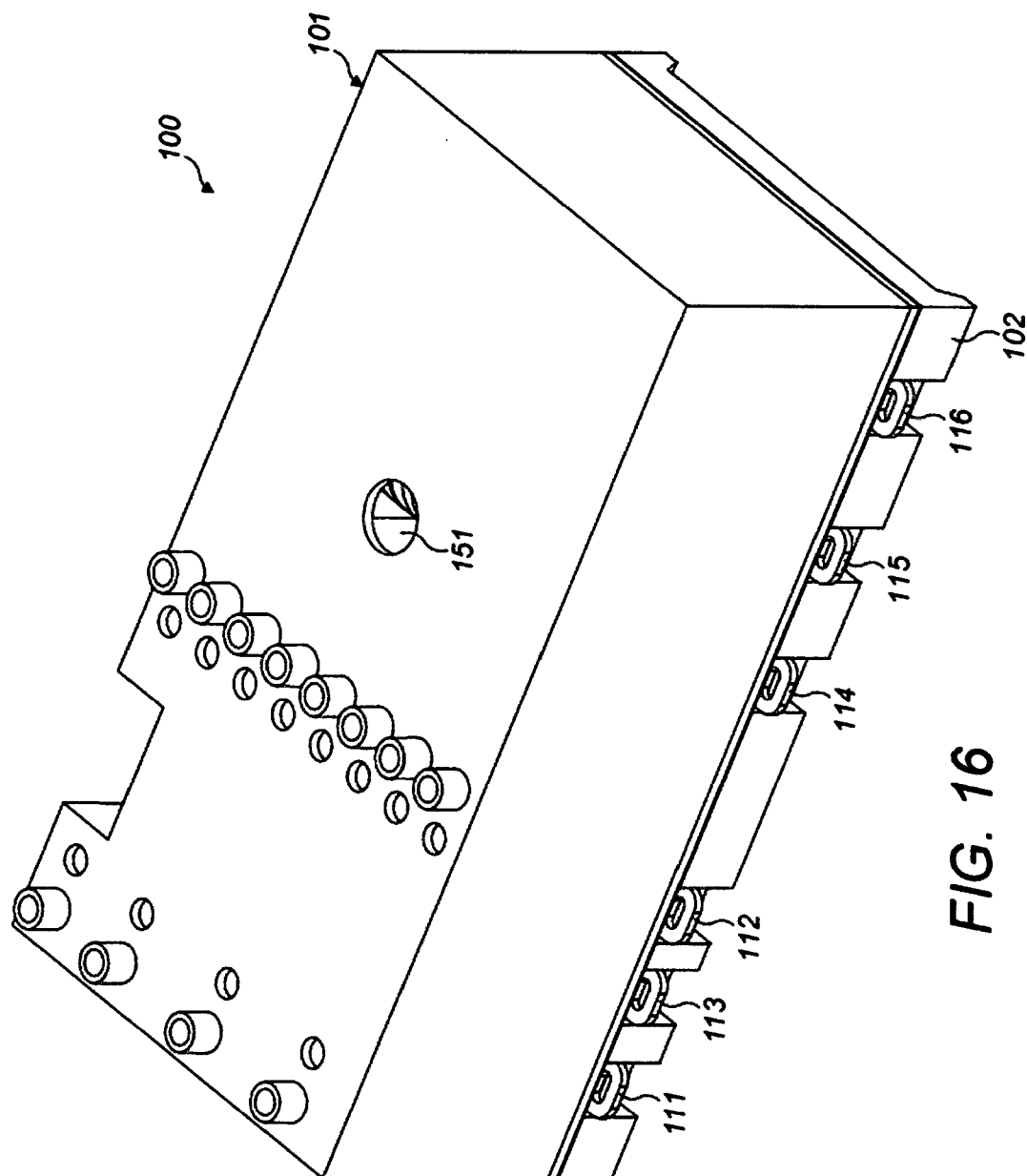


FIG. 14

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/051027

A. CLASSIFICATION OF SUBJECT MATTER INV. C12Q1/04 C12Q1/68 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A X	WO 2016/005768 A1 (MOMENTUM BIOSCIENCE LTD [GB]) 14 January 2016 (2016-01-14) whole document, in particular p. 8, l. 14 - p. 9, l. 34; p. 10, l. 1-3; p. 10, l. 6-19; p. 10, l. 21 - p. 11, l. 2; p. 12, l. 5-11; p. 12, l. 17-20; p. 30, l. 22-27; claims 1-58 ----- WO 2014/193481 A1 (KRISHNAMURTHY RAJESH [US]) 4 December 2014 (2014-12-04) whole document, in particular par. 13, 14, 19, 20, 34, 70, 77, 79-85, 88, 100-104, 109, 111, 113, 119, 120, 151-153; example 1, 2; fig. 1, 3 ----- -/-	6 1,10-15, 44,49, 51-57, 59,60 1,9,13, 15,44, 51,53, 57,59,60
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 16 June 2017		Date of mailing of the international search report 23/08/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Chrétien, Eva Maria

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2017/051027

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/023198 A2 (ZYBAC LLC [US]; ERICSON DANIEL G [US]; BRANDY KYLE R [US]) 14 February 2013 (2013-02-14)	1,9,44
Y	whole document, in particular p. 18, l. 12-24; p. 19, l. 6-9; p. 24, l. 24 - p. 25, l. 11; fig. 7, 8	6,10-15, 49, 51-57, 59,60
X	----- WO 2010/091080 A2 (NETWORK BIOSYSTEMS INC [US]; SELDEN RICHARD F [US]; TAN EUGENE [US]) 12 August 2010 (2010-08-12)	1,6, 9-15,44, 49, 51-57, 59,60
Y	whole document, in particular p. 10, l. 15-30; p. 11, l. 6-14; p. 13, l. 2 - p. 14, l. 2; example 1	10-15
Y	----- WO 2009/007719 A2 (ISEAO TECHNOLOGIES LTD [GB]; STANLEY CHRISTOPHER JOHN [GB]; WILSON STU) 15 January 2009 (2009-01-15) whole document, in particular p. 17, l. 18 - p. 19, l. 9; p. 23, l. 27 - p. 24, l. 4; p. 48, l. 15 - p. 53, l. 8; example 4; claims 1-19	6,49, 51-57, 59,60
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A	----- US 2013/309700 A1 (HEARN ANDREW [US] ET AL) 21 November 2013 (2013-11-21) whole document, in particular par. 19, 24-28, 40, 54, 55, 60, 61, 73, 74, 76, 82, 83	1,6, 9-15,44, 49, 51-57, 59,60
A	----- WO 2011/070507 A1 (KONINKL PHILIPS ELECTRONICS NV [NL]; VAN MEERBERGEN BART EDWARD GUSTA) 16 June 2011 (2011-06-16) whole document, in particular p. 7, l. 3-15; p. 9, l. 5-34 -----	1,6, 9-15,44, 49, 51-57, 59,60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2017/051027

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1(completely); 6, 9-15, 44, 49, 51-57, 59, 60(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1(completely); 6, 9-15, 44, 49, 51-57, 59, 60(partially)

A method of separating microorganisms from non-microorganism cells in a non-microorganism cell-containing sample, the method comprising: a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter.

2. claims: 2, 3(completely); 6, 7, 9-18, 24, 44-60(partially)

A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising: a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter d. detecting the absence or presence of microorganisms retained within or upon the filter.

3. claims: 4, 5, 8, 19, 20(completely); 6, 7, 9-18, 24, 44-60(partially)

A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising: a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample b. filtering the lysate through a first filter that prevents passage of insoluble cellular material through the filter c. filtering the filtrate from step b through a second filter that retains microorganisms within or upon the filter d. lysis of the microorganisms retained within the filter following step c e. incubating the lysate from step d with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and f. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism.

4. claims: 21-23(completely); 24, 44-60(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising: a. selective lysis of non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample b. separation of the intact microorganisms from the lysed cell material c. lysis of the microorganisms d. incubating the lysate from step c with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the microorganisms; and e. specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying enzyme on the substrate nucleic acid molecule to indicate the absence or presence of the microorganism; characterised in that step c is performed at a temperature of no more than around 30°C, optionally between around 15°C and 30°C or between around 18°C and 25°C, such as around 18, 19, 20, 21, 22, 23, 24 or 25°C.

5. claims: 25-42

A device, composition of matter or kit for detecting the absence or presence of a microorganism in a sample that may also contain non-microorganism cells comprising: a. a first reservoir for a first lysis reagent that selectively lyses non-microorganism cells in the sample whilst retaining intact any microorganisms present in the sample b. a second reservoir for a second lysis reagent that lyses microorganisms in the sample c. a sample inlet for addition of the sample to enable mixing of the sample with the first lysis reagent d. a filtration housing comprising: I. a first filter which captures insoluble cellular material (precipitated nuclear material) II. a second filter, downstream of the first filter, which captures microorganisms III. an outlet downstream of the second filter; and e. a fluid channel arrangement configured to provide selective fluid communication between the filtration housing and any one or more of the first reservoir, the second reservoir and the sample inlet.

6. claim: 43

A multi-channel device, composition of matter or kit comprising a plurality of the device, composition of matter or kit of any of claims 25 to 42 in parallel wherein the first and second filters of the plurality of the device or kit are located such that a net direction of flow of fluid through the multi-channel device or kit is parallel to the plane or planes of the first and second filters.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2017/051027

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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