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(54) Title: MATERIALS AND METHODS FOR CAPTURE OF PATHOGENS AND REMOVAL OF AURINTRICARBOXYLIC ACID FROM A SAMPLE

(57) Abstract: The present invention concerns methods and materials for extracting infectious pathogens from a sample, such as blood, comprising the steps of creating a fibrin aggregate confining the pathogens and introducing a fibrin lysis reagent to expose the pathogens for analysis. The fibrin lysis reagent is preferably composed of plasminogen and streptokinase frozen in coincident relation until the fibrin lysis reagent is needed whereby streptokinase enzymatically reacts with plasminogen to form plasmin upon thawing. Preferably, the plasminogen is suspended in an aqueous salt solution prior to freezing including NaCl and Na_3PO_4. The subject invention also concerns materials and methods for efficiently removing ATA from a sample comprising nucleic acid composition. The subject methods provide a nucleic acid composition sufficiently free of ATA such that a RT-PCR reaction and other reactions involving reverse transcriptase can be performed.

WO 2005/021799 A2

DESCRIPTION

MATERIALS AND METHODS FOR CAPTURE OF PATHOGENS AND REMOVAL OF AURINTRICARBOXYLIC ACID FROM A SAMPLE

The present invention was made with the support of the U.S. Army Solider and Biological Chemical Command under Grant No. DAAD13-01-C-0045. The Government may have certain rights to this invention.

Cross-Reference to Related Applications

This application claims the benefit of U.S. application Serial No. 10/604,779, filed August 15, 2003, and U.S. Provisional application Serial No. 60/481,892, filed January 14, 2004, the disclosure of each of which is incorporated herein by reference in its entirety.

Background of the Invention

The threat of bioterrorism (BT) and biological warfare presents challenges for the clinical setting that are best met with rapid and sensitive technologies to detect BT agents. Peripheral blood samples could contribute to early and specific clinical and epidemiological management of a biological attack if detection could take place when the concentration of the infecting organism is still very low. The worried well and recently infected patients would benefit, both psychologically and physically, from early pharmacological intervention.

Infection with *Bacillus anthracis* or *Yersinia pestis* often present initially as a nonspecific febrile or flu-like illness. The mediastinitis associated with inhalational anthrax ultimately results in bacilli entering the blood once the efferent lymphatics become laden with organisms. When bacteremia (the presence of bacteria in the blood) and sepsis (the invasion of bodily tissue by pathogenic bacteria) have initiated, the number of bacilli may increase quickly, doubling every 48 minutes, most often resulting in death of the patient.

It has been reported that microbiological studies on patient blood samples are useful for diagnosing pneumonic plague. The potential for *Yersinia pestis* bacilli to be present in peripheral circulating blood suggests that a PCR assay would make a useful diagnostic tool. Testing for pneumonic plague or inhalational anthrax would be effective when healthy patients present with "flu-like" symptoms (malaise, fever, cough, chest pain and shortness of breath) that may accompany other nonspecific symptoms. However, in order to maximize

the probability of successful, detection of the infecting organism must take place early in the disease process, when the concentration of circulating bacteria is very low.

Extraction of pathogen DNA from whole blood typically requires between 200µl to 500µl of patient sample for each preparation event. Detection of early bacteremia is improved by using an entire 6 ml tube of patient blood for a single sample preparation event. Prior art literature describes a single tube blood culture system exploiting the selective lysis of blood elements, followed by centrifugation to pellet bacteria for plating on solid media. The technique has been examined thoroughly in conjunction with microbiological testing.

Fibrin is an insoluble protein precipitated from blood that forms a network of fibers. In vivo, this process is central to blood clotting. Fibrin is created by the proteolytic cleavage of terminal peptides in fibrinogen. In the laboratory analysis of blood, an aggregate (pellet) of fibrin collects at the bottom of a tube when blood is centrifuged. Within the fibrin aggregate, pathogens are trapped. The analysis of these pathogens is highly desirable. However, like coins embedded in a slab of concrete, the captured pathogens are substantially hidden from analysis, trapped in the fibrin aggregate. For individuals potentially exposed to dangerous pathogens, time is of the essence and rapid identification of the captured pathogens is paramount.

Plasmin is a substance in blood capable of converting fibrin to fibrinogen monomers. Plasminogen is a precursor of plasmin in the blood. Streptokinase is an enzyme that activates plasminogen to form plasmin. The combination of plasminogen and streptokinase in the presence of the fibrin aggregate containing blood elements and bacteria (formally present in peripheral circulation) allows the conversion of the fibrin aggregate to a liquid state.

This conversion facilitates rapid and efficient pathogen analysis through blood culture, antibody based testing, or nucleic acid sequence based testing (Reverse Transcription PCR, PCR, NASBA, TMA or the like).

The addition of DNAse (a DNA nuclease) to the above-described reaction provides for the conversion of human DNA into short fragments. This conversion of human DNA into short fragments contributes to a more rapid and efficient protein hydrolysis process during DNA extraction. This conversion of human DNA into short fragments is done while the bacterial DNA is protected. The short fragment human DNA is carried less efficiently through the DNA extraction process and hence represents a smaller proportion of total DNA product. As a result, the reduced human DNA level presents less of an inhibitory component to the nucleic acid sequence based reactions.

Aurintricarboxylic acid (ATA) is a polymeric anion that has been demonstrated in the literature to be a potent ribonuclease inhibitor. The compound has been described previously as an additive to sample lysis buffers where the objective is to extract RNA species from tissue samples. The nucleic acid extract derived from such procedures has been shown to be suitable for hybridization and gel electrophoresis analysis. However, ATA is a potent inhibitor of reverse transcriptase, which is essential for the polymerase chain reaction (PCR) detection of RNA species. Published procedures to remove ATA from nucleic acid containing compositions have revolved around chromatographic procedures that eliminate or remove only a portion of the ATA.

The use of ATA in a proteinase K lysis buffer is potentially superior to 1) chaothrophic salts (since they tend to reduce the efficiency of proteinase K driven protein hydrolysis as evidenced by PCR results); 2) protein based ribonuclease inhibitors (since these inhibitors would be broken down by proteinase K); and 3) EDTA (which only indirectly inhibits nucleases via chelation of the divalent cations used by those nucleases). In fact, divalent cations must be added to RNA preparations where enzymatic DNA hydrolysis is conducted. What has not been demonstrated in prior art is a method where, once added, the complete downstream removal of ATA from nucleic acid extracts can be achieved to the point that down stream reverse transcriptase PCR (RT-PCR) will function.

Also not previously described in the art is a way to utilize ATA in a lyses buffer to treat a large volume (1-10ml) whole blood sample and after several reagents addition steps move directly to RNA array hybridization using the entire blood sample for one analysis event hence bypassing RNA extraction and amplification.

Also not previously described in the art is a way to use blood samples treated with ATA containing mixtures in combination with pathogen capture using bioactive peptides functionalized on hyaluronic acid detailed in U.S. application number 10/604,779 where the hyaluronic acid in turn acts as a polymeric waveguide.

Also not previously described in the art is a way to cause a calcium release at the site of pathogen capture via bioactive peptide or annealing of RNA species so as to trigger the conversion of reporter molecule labeled fibrinogen to insoluble fibrin at the site of pathogen capture via bioactive peptide or annealing of RNA species upon the matrix of the hyaluronic acid polymeric waveguide.

ATA also serves an important function in the protection of bacterial DNA when that bacteria is present in a blood sample processed with reagents containing high levels ($\geq$100

U/ml) of DNase I as is used in various embodiments contained within U.S. application number 10/604,779. In order to achieve RNA detection capabilities that are superior to what can be achieved with technology described in U.S. application number 10/604,779, and to do so without additional steps or requirements, the present invention is utilized in combination with blood sample treatment technology described in U.S. application number 10/604,779 and prior art nucleic acid extraction methods that utilize chaotrophic salts such as guanidine thiocyanate in the presence of capture matrices such as silica or methods that utilize precipitation methods to concentrate nucleic acids out of crude samples.

Accordingly, there remains a need in the art for: 1) a method of destroying and making soluble the spectrum of blood element components (erythrocytes, leukocytes, nuclear membranes, fibrin, and host nucleic acid) without damaging analyte particles (bacteria, virus, fungi, toxin, metabolic markers, disease state markers, or chemical agents) in order to expose and rapidly concentrate (via centrifugation, filtration, or capture) the analyte particles from large volumes of blood, 2) removal of the host DNA and the matrix associated biomass present in the large volume blood sample using a single step enzyme detergent cocktail that is amenable to automation and portable systems, and 3) an analyte particle concentration method that can be coupled to existing manual or automated processes for nucleic acid extraction, biosensor testing, or liquid chromatography separation and mass spectrometry analysis.

Brief Summary of the Invention

The present invention concerns methods and materials for extracting infectious pathogens from a volume of a sample, such as blood, and includes the steps of creating a fibrin aggregate confining the pathogens and introducing a fibrin lysis reagent to expose the pathogens for analysis and DNAse to facilitate DNA extraction. The fibrin lysis reagents may be composed of DNAse, plasminogen and streptokinase frozen in coincident relation until the fibrin lysis reagent is needed whereby streptokinase enzymatically reacts with plasminogen to form plasmin upon thawing and introduction into the fibrin sample. Preferably, the plasminogen is suspended in an aqueous salt solution prior to freezing including NaCl and Na_3PO_4. The fibrin lysis reagent is preferably composed of DNAse and Phospholipase A_2. The DNAse enzyme is used to facilitate the chemical and physical disruption of pelleted blood elements that result from the previously described protocol.

Phospholipase A_2 is used to help human DNA digestion by destroying phospholipid bilayers and, hence, destruction of the nuclear membrane.

The subject invention concerns materials and methods for efficiently removing ATA from a sample, such as a sample consisting of a nucleic acid composition. The subject methods provide a nucleic acid composition sufficiently free of ATA such that a RT-PCR reaction and other reactions involving reverse transcriptase can be performed.

The subject invention also concerns materials and methods for a mixture of ATA, magnesium chloride, potassium phosphate, and sodium chloride that is dried and combined with other dried components such as those described herein.

The subject invention also concerns materials and methods for heating a solution of urea, diethylenetriaminepentaacetate (DTPA), optionally containing EDTA, sodium citrate, and sodium chloride, to at least 600 ° C for 4 hours followed by drying and combination with proteinase K and optionally Methyl 6-O- (N-heptylcarbamoyl)-α-D-glucopyranoside and the use of this reagent to allow ATA removal from nucleic acid extracts made with existing prior art methods based on chaotrophic salts or nucleic acid precipitation followed by centrifugation or methods described herein to allow downstream hybridization of RNA species directly out of treated whole blood samples.

The subject invention also concerns the urea/DTPA reagent that was heat treated to above 600 ° C for 4 hours during production and used in sample treatment as described above followed by the combination of urease to break down the urea followed by RNA array analysis.

The subject invention also concerns materials and methods for pathogen capture using bioactive peptides functionalized on hyaluronic acid as described herein where the hyaluronic acid in turn acts as a polymeric waveguide.

The subject invention also concerns methods to cause a calcium release at the site of pathogen capture via bioactive peptide or annealing of RNA species so as to trigger the conversion of reporter molecule labeled fibrinogen to insoluble fibrin at the site of pathogen capture via bioactive peptide or annealing of RNA species upon the matrix of the hyaluronic acid polymeric waveguide.

The subject invention also concerns materials and methods where the hyaluronic acid matrix that is cross linked utilizing biotin and strepavidin and functionalized with bioactive peptides, such as those described herein, can be subsequently broken down with hyaluronidase in order to facilitate pathogen elution.

Brief Description of the Drawings

Figure 1 is a diagrammatic view of the method according to the invention according to the invention.

Figure 2 is a diagrammatic view of the preparation of the fibrin lysis reagent according to Protocol 1 of the invention.

Figure 3 is a table providing data on *Bacillus anthracis* blood protocol.

Figure 4 is a table providing data on a comparison of two blood samples from different individuals.

Figure 5 is a table providing data on an evaluation of the present method by a Department of Health laboratorian.

Figure 6 is a table providing data on *Yersinia pestis* blood protocol.

Figure 7 is a diagrammatic view of the setup of extraction reagents according to Protocol 1 of the invention.

Figures 7-9 are diagrammatic views of bacterial recovery and fibrin lysis according to Protocol 1 of the invention.

Figure 10-13 are diagrammatic views of bacterial lysis and nucleic acid extraction according to Protocol 1 of the invention.

Figure 14a is a diagrammatic view of the steps of extracting reagents according to Protocol 2 of the invention.

Figure 14b is a diagrammatic view of the steps of extracting reagents according to Protocol 2 of the invention.

Figure 15 is a diagrammatic view of the steps of extracting reagents according to Protocol 3 of the invention.

Figure 16a is a diagrammatic view of the steps of extracting reagents according to Protocol 4 of the invention.

Figure 16b is a diagrammatic view of the steps of extracting reagents according to Protocol 4 of the invention.

Figure 17 is a table providing data on noise band crossing points for blood samples spiked with *B. anthracis* and processed with plasminogen, streptokinase, phospholipase A_2, DNase I, and lipase with centrifugation or filtration.

Figure 18 shows sedimentation and solublization of tissue aggregates from 6 ml blood samples exposed to various detergent and enzyme treatments.

Figure 19 shows filtration characteristics of 6 ml blood samples exposed to various detergent and enzyme treatments.

Detailed Disclosure of the Invention

The present invention concerns methods of extracting infectious pathogens from a biological sample, such as a volume of blood, and includes the steps of creating a fibrin aggregate confining the pathogens and introducing a fibrin lysis reagent to expose the pathogens for analysis and DNAse to facilitate DNA extraction. The fibrin lysis reagents may be composed of DNAse, plasminogen and streptokinase frozen in coincident relation until the fibrin lysis reagent is needed whereby streptokinase enzymatically reacts with plasminogen to form plasmin upon thawing and introduction into the fibrin sample. Preferably, the plasminogen is suspended in an aqueous salt solution prior to freezing including NaCl and Na_3PO_4. The fibrin lysis reagent is preferably composed of DNAse and Phospholipase A_2. The DNAse enzyme is used to facilitate the chemical and physical disruption of pelleted blood elements that result from the previously described protocol. Phospholipase A_2 is used to help human DNA digestion by destroying phospholipid bilayers and, hence, destruction of the nuclear membrane.

The present invention utilizes resuspension of the dried enzymes in a buffer solution using Potassium Phosphate as an aide to blood element solublization. It is imperative that the streptokinase and plasminogen are not mixed with the buffer solution until immediately prior to the addition of the blood sample. The Potassium Phosphate pH range is about 7.8 to 8.0, which is different from prior art that claims an effective pH range of 7.2 to 7.6. Prior art uses phosphate ion solutions with lower pH to act as a true buffer; however, the current method allows for optimal Phospholipase A_2 activity and Magnesium solubility. Magnesium is present in the buffer solution as the divalent cation driving the activity of Phospholipase A_2 in the presence of DNase. Prior art uses calcium as the classic divalent cation for driving Phospholipase A_2 activity, however, calcium is not compatible with the phosphate ions essential for blood element solublization.

An embodiment of the present invention includes concentrating and extracting particles such as prions, toxins, metabolic markers, cancerous matter, disease state markers, bacteria, virus, and fungi from a volume of blood by introducing an enzyme-detergent combination to expose pathogens in the blood sample and analyzing the blood sample for the particles now readily identifiable via the extraction. The enzyme-detergent may be a fibrin

lysis reagent comprising plasminogen and streptokinase. The plasminogen and streptokinase may be frozen in coincident relation until the fibrin lysis reagent is needed. The streptokinase then reacts with the plasminogen to form plasmin upon thawing. The plasminogen may be suspended in an aqueous salt solution prior to freezing. Suitable salt solutions may include NaCl, $NaPO_4$ or the like. To enhance analysis, the particles may be replicated via polymerase chain reactions (PCR).

By introducing DNase, the process is facilitated by the conversion of DNA into short fragments thereby contributing to a more rapid and efficient protein hydrolysis process during DNA extraction and lowering the burden of inhibitory human DNA. Similarly, introduction of Endonuclease produces a similar advantage.

As an alternative to freezing, the enzyme-detergent may include dried streptokinase and dried plasminogen as the fibrin lysis reagents. The dried reagents may then be mixed and distributed into disposable test containers. This embodiment may be particularly useful for field-testing in locations where sophisticated laboratory equipment and controls are unavailable.

The plasminogen may be combined with Phospholipase A2. DNase, Endonuclease, Lipase, and combinations thereof. The dried enzyme-detergent combination may be suspended in pellets of trehalose buffer and packaged into tubes as a dry reagent. The dried reagents may then be resuspended in a buffer, added to a 1-10ml volume of blood and incubated for about 5-20 minutes at room temperature. More specifically, the dried reagent is comprised of about 1,500-4,500 KU Phospholipase A2, about 5,000-10,000 U Streptokinase, about 2-10 U Plasminogen, about 200-3,650 U DNase, about 200-4,000 U Endonuclease, and about 10,000-100,000 Lipase.

The solution may be centrifuged for approximately 20 minutes at 5,000-5,500 x g at a temperature of 10-20°C, the supernatant decanted, and the pellet washed. The pellet may be washed three times with a 10-20 mM solution of Ecotine/20 mM HEPES ph 7.7 and/or a 10-20 mM solution of sucrose/20 mM HEPES ph 7.7. The resultant sample may then be applied to a commercially available nucleic acid extraction method.

Digesting the sample may include lysis and DNase inactivation or lysis and Endonuclease inactivation. 12.5-25 mg proteinase K, 1-105% SDS (sodium dodecyl sulfate), 10-200 mM aurintricarboxylic acid, and 10- 20mM sodium citrate buffer pH 7.8-8.4 may be utilized, the solution allowed to incubate at room temperature for 10 minutes. The sample may then be filtered with a 0.22-0.45 μm filter unit, washed with a 10-200 mM

Aurintricarboxylic Acid, digested with lysis and DNase inactivation and/or Endonuclease inactivation, and purified.

Digesting the sample may include the steps of combining 12.5-25 mg proteinease K, 1-1.5% SDS, 10-200 mM aurintricarboxylic acid, and 10-20 mM sodium citrate buffer, incubating at room temperature for 10 minutes, and eluting the lysate from the filter surface by addition of 3.5-4.2 M guanidine isothiocyanate pH 6.4.

The solutions may be applied directly to a biosensor device wherein, responsive to the presence of the pathogens in the blood sample, the patient develops pathogenic or native disease state markers that allow for the capture and detection of these markers by the biosensor device. Alternatively, the solution may be applied directly to a liquid chromatography mass spectrometry device whereby, responsive to the presence of the pathogens in the blood sample, the patient develops pathogenic or native disease state markers that allow for the detection of mass signatures associated with the structural components of the pathogens using the mass spectrometry device.

The buffer can comprise detergent and salts. This may be achieved by aiding blood element solublization by introducing 10-30 mM Potassium Phosphate at a pH range of 7.8 to 8.0, driving Phospholipase A2 activity by adding 10-80 mM Magnesium Chloride as the divalent cation, adding 20-150 mM Sodium Chloride, and including 10-200 mM Aurintricarboxylic Acid during the DNase incubation process. The buffer may also include 1.0-1.2% Triton X-100. Additional steps may include combining 20-35 mM methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside and 0.05-0.1% Saponin; and storing the enzymes by using a trehalose buffer. Storing the enzymes is accomplished by using a trehalose buffer in combination with methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside. The trehalose storage buffer comprises 10 mM Potassium Phosphate, 0.01-0.04% Triton X-100, 1-5 mM Dithiothreitol, and 0.3-0.5 M Trehalose.

In Figure 1, a blood draw **30** is performed on a patient. A solution of PBS, pH 7.4 and 1.2% Triton X-100 is added, the blood is vortexed and centrifuged **40** creating pellet **60** in a 15 ml tube **50**. Preferably, resins, metal hydroxides, and/or nano materials may be added with the PBS/Triton X-100 solution to capture particles such as bacteria, virus, fungi, cancerous cells, prions, toxins and the like to contribute greater density to these particles. The increase in particle density allows lower speeds to run during centrifugation.

The supernatant is decanted leaving a fibrin aggregate. A fibrin lysis reagent **70** of the invention is added to tube 50 dissolving the fibrin aggregate and leaving pathogens 65

exposed for analysis. Pathogens **65** are vortexed, centrifuged, and subject to lysis to extract the pathogen DNA. The DNA is then replicated **90** and analyzed **100** for the identity of the suspected pathogen.

In an alternative embodiment of the invention, a device would be used to obviate the need for a centrifuge. The device will use flexible electrodes similar to a fish gill to collect particles (such as bacteria, virus, cancerous cells, prions, or toxins). The electrodes will also be used to collect resins and nano materials that have these particles attached to them. The device will resemble a bubble on a surface. An electrical potential will be used to accelerate pathogen capture. The device can be compressed to allow efficient removal of the contents. The device would preferably have the following properties: (1) a rigid base layer and flexible top layer; (2) flexible gills to be mounted on either the top or bottom layer; (3) Strepavidin and hyaluronic acid strands functionalized with bioactive peptides, antibodies, aptomers, molecular imprinted polymers, or metals that attract particles such as bacteria, virus, fungi, toxins, metabolic markers, disease state markers, or chemical agents are to be deposited on the flexible gill electrodes; (4) the flexible layer will have electrodes deposited on it; (5) counter electrodes for the gill electrodes will reside on the opposite side; (6) the average dead volume of the device is 300 micro liters – it is preferred that there is to be no residual material in the device after squeezing out the material from the device; and (7) polyimide will form the flexible portion and the electrodes will be made of Pt, Au, or carbon. The device is preferably used as follows: (1) flow liquid into the device and apply voltage at this time; (2) add chemicals and heat the device; and (3) squeeze out the device to remove all contents. The device is used to prepare a sample for analysis of particles (such as bacteria, virus, cancerous cells, prions, or toxins) using spectrophotometric, mass spectroscopy, antibodies, culture, or nucleic acid (e.g. PCR, NASBA, TMA) based detection systems.

A filtering device may be used to filter out the particles from blood treated with the Triton X-100 / PBS/ magnesium solutions with enzymes selected from the group of streptokinase, plasminogen, phospholipase A_2, DNase, and lipase. A filtering device may also be used to filter out the particles from blood treated with a combination of methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside, Saponin, and PBS / magnesium plus enzymes selected from the group of streptokinase, plasminogen, phospholipase A_2, DNase, and lipase. After washing away the enzyme and detergent treatment reagents and any residual broken down blood components, the particle is ready for analysis or further processing.

The preparation of one embodiment of a fibrin lysis reagent is shown as Protocol 1 in Figure 2 wherein NaCl, MnCl, DTT, DNAse, and plasminogen are added to mixing tube **110**. Sodium phosphate is then added to mixing tube **110** and the solution is distributed into 1.5 ml reagent tubes **120** placed on ice. The reagent tubes **120** are frozen to -75°C for approximately 20 minutes. Approximately 2,700 U of streptokinase **130** is added to the wall of reagent tubes **120** just above the frozen plasminogen solution.

Figures 3-6 provide PCR results derived from testing blood samples seeded with encapsulated vegetative avirulent *Bacillus anthracis* were grown according to CDC protocol # CDC.DFA.1.2, stored in 15% glycerol TSB, and frozen at -75°C. Stocks of avirulent *Yersinia pestis* grown in TSB at 37°C, frozen in 15% glycerol TSB, and frozen at -75°C. Bacterial counts were tested at the time of harvest and retested at the time of sample spike.

Figures for average *Bacillus anthracis* CFU per six ml of human blood are derived from post-freezing testing given the large standard deviation encountered in side-by-side post freezing dilution events. No significant cellular death is recognized or expected. A 30% cellular death rate is the highest that is reasonably expected in the worst circumstances. A conservative approach would be to increase all calculated *Bacillus anthracis* CFU by 30%.

Figures for average *Yersinia pestis* CFU per six ml of blood are derived from pre-freezing testing. The low standard deviation of pre-freezing count replicates and concordance with post-freezing testing allows use of the pre-freezing bacteria count numbers. This is a conservative approach that can be utilized given the now predictable results that are derived from storing and diluting this organism.

The present invention reproducibly generates analyte DNA appropriate for PCR testing of pathogens, such as *Bacillus anthracis,* using patient blood samples that are up to 3 months old. Sensitivity is 100% at <10 CFU / ml of human blood when using 6 ml of blood collected in a Becton Dickinson Vacutainer (Tables 1 and 2). This protocol also allows detection of *Yersinia pestis* at 100% sensitivity at <10 CFU / ml for at least one of four oligo sets according to the more limited data gathered for this organism (Table 3). It should be noted that CDC does not consider samples positive for *Y. pestis* unless two oligo sets produce an acceptable PCR signal.

In accordance with Protocol 1, Figure 7 shows a preferred method of the setup of extraction reagents according to the invention. Figures 8-9 show a method of bacterial recovery and fibrin lysis according to the invention. Figures 10-13 show a preferred method of bacterial lysis and nucleic acid extraction according to the invention.

In an alternative embodiment, as shown in Figures 14-16b, the individual enzymes of streptokinase and plasminogen are made into dried powders, mixed, then distributed to disposable tubes. In another embodiment, Phospholipase A_2, plasminogen, DNase or Endonuclease, and lipase are suspended and dried in pellets of trehalose buffer. Although Phospholipase A_2 is preferred, any enzyme that will destroy nuclear membrane while keeping bacterial cell wall or viral coats intact may also be used. Streptokinase is likewise suspended and dried in pellets of trehalose buffer. At least one pellet of the plasminogen and one pellet of the streptokinase are packaged into tubes as dried reagents.

Dried reagents of the invention can be resuspended in a 10 ml buffer solution comprising about 10-30 mM Potassium Phosphate, about 10-80 mM Magnesium Chloride, about 20-150 mM Sodium Chloride, about 10-200 mM Aurintricarboxylic Acid and about 1.0-1.2% Triton X-100. Aurintricarboxylic Acid is evidenced to provide a level of protection to bacterial nucleic acid without impeding human DNA digestion. The use of Aurintricarboxylic Acid is not described in prior methods of human DNA digestion. Methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside and Saponin can be substituted for Triton X-100. In one embodiment, the methyl 6-O-(N-heptylcarbomoyl)-α-D-glucopyranoside is used at 20-35 mM and the saponin is used at 0.05-0.19 concentration. The methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside is stored with the phospholipase A_2, plasminogen, DNase I, and lipase in a Trehalose storage buffer. Substitution of the Triton X-100 with the methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside and saponin solution allows for the efficient activity of Phospholipase A_2, provides the action of breaking up protein aggregates without denaturation, and is more genial to bacterial walls than Triton X-100. Use of Saponin and methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside in this combination is not described in the prior art. The Trehalose storage buffer can comprise 10 mM Potassium Phosphate pH 7.4, 0.01-0.04% Triton X-100 or methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside, 1-5 mM Dithiothreitol, and 0.3-0.5 Trehalose. The buffer and enzyme mix are then immediately combined with a 10 ml blood sample, which may be scaled down to 1 ml. The sample is then incubated at room temperature for 5-10 minutes. The aforementioned components aide blood element solublization through minimizing certain particulates that would otherwise clog filters, impair biosensors or mass spectrometry devices, and impede nucleic acid extraction. Solublization occurs while human DNA is efficiently digested and as viral and/or bacterial DNA remain intact.

In accordance with Protocol 2 and 4, a preferred enzyme combination is comprised of Streptokinase, Plasminogen, DNase or Endonuclease, Phospholipase A_2, and Lipase. Alternatively, enzyme combinations comprising of Streptokinase, Plasminogen, DNase or Endonuclease, and Phospholipase A_2 may also be used. In another alternative combination, Streptokinase, Plasminogen, DNase or Endonuclease may be used, as well as, DNase or Endonuclease, Phospholipase A_2 and Lipase. DNase or Endonuclease in combination with Phospholipase A_2 is yet another alternative. The efficacy of the three latter combinations was found to be equal.

In accordance with Protocol 3, a preferred enzyme combination is comprised of Streptokinase, Plasminogen, DNase or Endonuclease, Phospholipase A_2 , and Lipase. Alternatively, enzyme combinations comprising of Streptokinase, Plasminogen, DNase or Endonuclease, and Phospholipase A_2 may also be used. In another alternative combination, Streptokinase, Plasminogen, DNase or Endonuclease may be used.

As shown in Figure 14 with Protocol 2, the sample is centrifuged for a period of about 20 minutes at 5,000-5,500 x g at a temperature between 10-22°C after incubation. The supernatant is then decanted and the pellet washed three times with a 10-20 mM solution of Ecotine/20 mM HEPES pH 7.7 and/or a 20-30 mM solution of Sucrose/20 mM HEPES pH 7.7.

Alternatively after incubation, the Protocol 2 sample is centrifuged in similar fashion and the supernatant decanted, followed by sample lysis and DNase or Endonuclease inactivation using about 12.5-25 mg Proteinase K, about 1-1.5% Sodium Dodecyl Sulfate (SDS), about 10-200 mM Aurintricarboxylic Acid and about 10-20 mM Sodium Citrate buffer pH 7.8-8.4. The sample is allowed to incubate at room temperature for about 10 minutes. The digested sample may then be applied to any commercially available nucleic acid extraction method, shown in Figure 14b.

Yet in another alternative, referred to as Protocol 3 and depicted in Figure 15, the sample is filtered with a 0.22-0.45 μm filter unit and washed with 10-20 ml of about 10-200 mM Aurintricarboxylic Acid, followed by sample lysis and DNase or Endonuclease inactivation. Sample lysis and DNase or Endonuclease inactivation is accomplished by using about 12.5-25 mg Proteinase K, about 1-1.5% SDS, about 10-200 mM Aurintricarboxylic acid, and about 10-20 mM Sodium Citrate buffer. The sample is then incubated at room temperature for about 10 minutes. Addition of about 3.5-4.2 M Guanidine Isothiocyanate pH

6.4 is necessary to elute the lysate from the filter surface. The nucleic acid extract may then be further purified using a commercially available method.

Another alternative, referred to as Protocol 4 and shown as Figure 16a, applies the sample directly to a biosensor device that will capture and detect bacteria, virus, fungi, toxins, prions, chemical agents, metabolic markers or native disease state markers developed by the patient's own body in response to these pathogens and agents present in the blood sample.

In yet another Protocol 4 alternative shown in Figure 16b, the sample is applied directly to a liquid chromatography mass spectrometry device that will detect mass signatures of structural components that comprise bacteria, virus, toxins, prions, and chemical agents present in the blood sample or native disease state markers developed by the patients own body in response to these pathogens and agents present in the blood sample. It will be seen that the objects set forth above, and those made apparent from the foregoing description, are efficiently attained and since certain changes may be made in the above construction without departing from the scope of the invention, it is intended that all matters contained in the foregoing description or shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

The subject invention concerns materials and methods that can be used for the selective removal of ATA from a sample, such as a blood sample containing nucleic acid. Typically, ATA is used in procedures for extracting and purifying RNA from cells, viruses, *etc.*, because of its activity as a ribonuclease inhibitor. In one embodiment of the claimed invention, the potent ribonuclease inhibitor ATA will be present during the portion of the nucleic acid extraction process where protein hydrolysis is allowed to proceed at optimal conditions (*i.e.,* with ATA and not chaotrophic salts such as guanidine thiocyanate). Compositions for removing ATA can be provided in either solution form or dry, solid form. Preferably, compositions are provided in a dry solid form to which a liquid or fluid is subsequently added. In an exemplified embodiment, a composition of the invention is used in combination with the lysis reagents described herein.

In one embodiment, a method of the invention comprises contacting a sample that comprises ATA and, optionally, nucleic acid, with a urea/DTPA composition of the invention. In one embodiment, the sample can comprise any combination of reagents as described in a lysis buffer of the invention. A urea/DTPA composition of the invention can be prepared by combining urea with DTPA and optionally EDTA, sodium citrate, and enough of a base, such as sodium hydroxide to achieve pH 8.0 as defined in Table 1. In one

embodiment, the mixture is heated to above about 600 ° C for about 4 hours, dried, ground to a powder, and optionally combined with proteinase K and methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside. The urea/DTPA reagent is preferably provided in a dried form so as to minimize the downstream sample volumes and obviate the procedure of having to add proteinase K (PK) in a separate step (since PK is not stable for long periods of time in 6.0 to 7.5 M urea). The ground urea/DTPA reagent is dried under vacuum and added (at 360 mg / ml blood sample) to blood treated previously with lysis reagents described herein. The sample treated with urea/DTPA is incubated about 5 – 10 minutes at about 65 ° C. When samples treated with ATA and a urea/DTPA reagent of the invention are combined with prior art nucleic acid extraction protocols where binding matrices such as silica or other materials that bind nucleic acids in the presence of chaotrophic salts or where precipitation and centrifugation is used, the ATA will not co-purify with the nucleic acid extract. In another embodiment, the proteinase K can be inactivated by exposure to temperatures above about 80 ° C for 5 – 10 or more minutes, the sample then cooled to below about 40 ° C, wherein urease is then added to about 1,000 – 100,000 U / ml to break down the urea. The sample can then be applied directly to a nucleic acid array device.

Using the subject methods in conjunction with PCR, 10 CFU *Bacillus anthracis* per 10 ml of blood can be detected. Also, there was no difference in the RT-PCR kinetics derived from PBS samples where 1 ng of MS2 RNA was seeded into nucleic acid extracts made with and without ATA. Also, 1,000 pfu polio sabin III virus / 8 ml SPS whole blood was detected by RT-PCR when lysis reagents described herein were combined with the urea/DTPA reagent and protocol described above. By using a urea/DTPA reagent of the invention, ATA that was present prior to the proteinase K digestion step did not have a negative impact on the PCR kinetics using the nucleic acid extracts that were prepared using the subject methods.

Table 1. Contents of powdered Urea/DTPA reagent (upon addition of 1 ml sample to 360 mg reagent
Urea 6.0 – 7.5 M
Methyl 6-O- (N-heptylcarbamoyl)-α-D-glucopyranoside 10 – 20 mg / ml
Proteinase K 600 – 1,000 μg / ml
EDTA 20 – 70 mM
DTPA 20 – 70 mM
Sodium Citrate 120 mM
Sodium Hydroxide add to pH 8.0

In another embodiment, if a sample is not processed with lysis reagents, such as those described herein, then a buffer comprising only ATA can be added to the cells as a first step and subsequently treated as outlined above.

In another embodiment, urea can be added to about 6.0 – 7.5 M to an ATA containing sample, and then combined with prior art chaotrophic salt based binding buffers and silica binding matrices, conduct the protocol according to the literature citation or manufacturer specifications with the exception of heating the chaotrophic salt based binding and wash buffer to about 55 – 65 ° C prior to use with the sample. The reaction of urea with the aurintricarboxylic acid (ATA) plus the combination of this solution with chaotrophic salt at about 55 - 65 ° C followed by application to a silica based nucleic acid capture matrix allows the selective binding of nucleic acid to the matrix and exclusion of ATA (passed out in the column flow through). It is the combination of reaction with urea and heat that provides for the exclusion of ATA from the silica capture matrix while nucleic acid binds readily. The above described urea/DTPA reagent produced by heating above 600 ° C during production eliminates the need for this chaotrophic salt heat step and allows for more complete removal of the ATA.

In another embodiment, blood samples can be treated with ATA containing mixtures described herein combination with pathogen capture using bioactive peptides functionalized on hyaluronic acid also as described herein where the hyaluronic acid in turn acts as a polymeric waveguide. The hyaluronic acid is labeled with biotin via carboxyl groups or amines and the excess unbound biotin is subsequently removed via dialysis. Strepavidin is cross-linked and the excess unbound cross linker is removed via dialysis. The cross-linked

strepavidin is added in 100 – 10,000 molar excess to the biotinylated hyaluronic acid and incubated about 4 – 10 hours with or without an applied electrophoretic or dielectrophoretic field. Alternatively, the strepavidin is added in the described ratios, incubated for about 1 – 4 hours with mixing, combined with a photo- activated cross-linking reagent, and cross-linked within as lithography system in order to generate structures positioned within a sample flow path. In this system a calcium release at the site of pathogen capture via bioactive peptide or annealing of RNA species is in the presence of thrombin (about 10 – 500 μg per milliliter) used to trigger the local conversion of reporter molecule labeled fibrinogen (about 10 – 500 μg per milliliter) to an insoluble fibrin aggregate at the site of pathogen capture via bioactive peptide or annealing of RNA species upon the matrix of the hyaluronic acid polymeric waveguide. Reporter molecules can be any molecule that can be detected and include, for example, fluorescent molecules (fluorescein, *etc.*), radioactive molecules, enzymes, antigens, and the like. As used herein, bioactive peptides include native and modified non-specific virus binding peptides most optimally, such as lactoferrin or fatty acid modified lactoferrin, and native and modified non-specific bacteria binding peptides, most optimally, such as Cecropin P1, but also including, for example, protamine, Buforin I, Buforin II, Defensin, D-Magainin II, Cecrpin A, Cecropin B, Lectin PA-1, and Tritrpticin. The modified peptides may be altered in terms of amino acid content and include the salts, esters, amides, and acylated forms thereof.

In another embodiment, the bioactive peptides functionalized upon the hyaluronic acid (that is cross linked via biotin and strepavidin) act as pathogen capture moieties. Upon pathogen or biomarker capture, the hyaluronic acid is broken down using about 1,000 – 1,000,000 units of hyaluronidase / ml of sample within the device.

In another embodiment, the ATA, magnesium chloride, and potassium phosphate components described in the lysis buffer of the present invention are combined, brought to about pH 9.2 – 10 in batches of 100 ml, and heated to boiling until a dry residue forms. The dry residue is ground up, dried further under vacuum, and added to the other enzyme, detergent, and Trehalose components, such as those described herein.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

I claim:

1. A method for removing ATA from a sample, comprising contacting the sample with a composition comprising urea and bringing the pH of the sample to about 8.0.

2. The method according to claim 1, wherein said composition further comprises sodium citrate.

3. The method according to any preceding claim, wherein said composition further comprises proteinase K.

4. The method according to any preceding claim, wherein said composition further comprises methyl 6-O- (N-heptylcarbamoyl)-α-D-glucopyranoside.

5. The method according to any preceding claim, wherein the pH is brought to about 8.0 by sodium hydroxide.

6. The method according to any preceding claim, wherein said composition further comprises EDTA or DTPA or both EDTA and DTPA.

7. A method of removing aurintricarboxylic acid from samples to facilitate efficient subsequent nucleic acid extraction or direct RNA array analysis, the method comprising: exposing a whole blood sample to reagents detailed in U.S. application number 10/604,779; and subsequently adding a urea containing solution prior to subsequent processing such as nucleic acid binding to a matrix in the presence of a chaotrophic salt or precipitation of nucleic acid followed by precipitation.

8. The method according to claim 7, where the addition of urea to an ATA containing sample will provide for ATA removal when classic chaotrophic salts and binding matrices such as silica are used for nucleic acid extraction and where the chaotrophic salt is heated between 55 – 65 ° C during washing and or binding.

9. The method according to claim 7, where the heating of a urea and DTPA solution during production to at least 600 ° C for 1- 4 hours, grinding to a powder; and adding directly to ATA containing samples followed by any nucleic acid extraction method.

10. The method according to claim 9, further comprising the step of adding one or all of the following compounds during production of the urea/DTPA reagent; adding EDTA and or sodium citrate and or, sodium hydroxide.

11. The method according to claim 9, further comprising the step of adding proteinase K to the dried powder consisting of urea and DTPA and one or all of the compounds in claim 10.

12. The method according to claim 9, further comprising the step of adding methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside to the dried powder consisting of urea and DTPA and one or all of the compounds in claim 10 and 11.

13. The method according to claim 9, further comprising the step of adding urease 1,000 – 10,000 U / ml to the sample after treatment with the urea/DTPA reagents that can be derived from any combination according to claims 9, 10, 11, and/or 12.

14. The method according to claim 9, further comprising the step of hybridizing RNA directly out of the whole blood sample (treated first with ATA and reagents listed in U.S. application number 10/604,779, secondly the urea/DTPA, and lastly urease) and onto DNA oligos functionalized onto a hyaluronic acid matrix that is cross linked with biotin and strepavidin.

15. A method where an insoluble form of calcium is labeled onto a protein such as a mutated RNase H that in turn bonds to but does not degrade RNA/DNA hybrids; and said protein carries an insoluble form of calcium to the site of wild type RNA binding to complementary DNA oligos functionalized on the cross linked hyaluronic acid matrix.

16. The method according to claim 14, where an insoluble form of calcium is labeled onto proteins such as bioactive peptides that aggregate on the outside of bacterial cell

walls or viral protein structures and said protein carries an insoluble form of calcium to the site of bacterial binding to bioactive peptides functionalized on a hyaluronic acid matrix.

17. The method according to claim 14, where the localized calcium is made soluble and hence released by an electrical potential induced local pH shift close to the surface of the cross linked hyaluronic acid structure.

18. The method according to claim 14, where fibrinogen labeled with reporter molecules that allow flourimetric, colorimetric, electrochemical, electromagnetic, or potentiometric detection is present in solution with thrombin when a local pH shift is generated by an induced electrical potential; and the fibrinogen is converted to insoluble fibrin by the activity of thrombin in the presence of calcium being released at the site of bacterial, viral, or RNA binding to bioactive peptides or DNA oligos respectively.

19. The method according to claim 14, further comprising the step where the cross linked hyaluronic acid acts as a waveguide for optical and electronic emissions.

20. The method according to claim 14, further comprising the step where the cross linked hyaluronic acid acts as a waveguide for optical and electronic emissions resulting from the aggregation of labeled fibrin; and where the conversion of labeled fibrinogen to fibrin aggregated has been precipitated by the release of soluble calcium in the presence of thrombin.

21. The method of clam 14, where the RNA array is composed of cross linked hyaluronic acid posts extending into a flow stream of treated blood sample; and where signals are read from the ends of the hyaluronic acid waveguide posts.

22. A method of using hyaluronic acid cross linked with biotin and strepavidin and functionalized with bioactive peptides used to capture pathogens (as described in U.S. application number 10/604,779) where the hyaluronic acid deposited inside a pathogen capture device is subsequently broken down via hyaluronidase so that the sample may be extruded out.

23. A method of concentrating and extracting particles from a blood sample, the method comprising:

exposing the blood sample to an enzyme-detergent combination; and

analyzing the exposed blood sample for the presence of particles.

24. The method according to claim 23, wherein the enzyme-detergent combination comprises plasminogen and streptokinase.

25. The method according to claim 24, further comprising the steps of:

freezing the plasminogen and streptokinase in coincident relation until a fibrin lysis reagent is needed; and

reacting streptokinase with plasminogen upon thawing whereby plasmin is formed.

26. The method according to claim 24, further comprising the step of suspending the plasminogen in an aqueous salt solution prior to freezing.

27. The method according to claim 26, wherein the aqueous salt solution comprises NaCl.

28. The method according to claim 26, wherein the aqueous salt solution comprises $NaPO_4$.

29. The method according to claim 23, wherein the particles are selected from a group consisting of prions, toxins, metabolic markers, cancerous matter, disease state markers, bacteria, virus, and fungi.

30. The method according to claim 23, further comprising the step of replicating the particles through PCR.

31. The method according to claim 23, further comprising the step of introducing DNase to the blood sample.

32. The method according to claim 23, further comprising the step of introducing Endonuclease to the blood sample.

33. The method according to claim 24, wherein the plasminogen and streptokinase are in a dried state.

34. The method according to claim 33, wherein the plasminogen and streptokinase are mixed and distributed in disposable test containers.

35. The method according to claim 33, wherein the plasminogen is combined with Phospholipase A_2, DNase, Endonuclease, and Lipase.

36. The method according to claim 35, wherein the enzyme-detergent combination is suspended then dried in pellets of trehalose buffer and packaged as a dry reagent.

37. The method according to claim 33, wherein the streptokinase is suspended then dried in pellets of trehalose buffer and packaged into tubes as a dry reagent.

38. The method according to claim 33, further comprising:
resuspending the dried reagents in a buffer;
adding the solution to the volume of blood; and
incubating the sample for at room temperature.

39. The method according to claim 38, wherein the dried reagent is comprised of about 1,500-4,500 KU Phospholipase A_2, about 5,000-10,000 U Streptokinase, about 2-10 U Plasminogen, about 200-3,650 U DNase, about 200-4,000 U Endonuclease, and about 10,000-100,000 Lipase.

40. The method according to claim 38, further comprising:
centrifuging the solution;
decanting the supernatant; and
washing the pellet.

41. The method according to claim 40, wherein the solution is centrifuged for approximately 20 minutes at 5,000-5,500 x g at a temperature of 10-20°C.

42. The method according to claim 40, wherein the pellet is washed with an Ecotine-HEPES solution.

43. The method according to claim 40, wherein the pellet is washed with a Sucrose-HEPES solution.

44. The method according to claim 40, wherein the pellet is washed with an Ecotine-HEPES solution and a Sucrose-HEPES solution.

45. The method according to claim 38, further comprising:
centrifuging the solution;
decanting the supernatant;
digesting the sample; and
applying the sample to a commercially available nucleic acid extraction method.

46. The method according to claim 45, wherein digesting the sample further comprises lysis and DNase inactivation.

47. The method according to claim 45, wherein digesting the sample further comprises lysis and Endonuclease inactivation.

48. The method according to claim 45, wherein digesting the sample further comprises utilizing proteinase K, sodium dodecyl sulfate, aurintricarboxylic acid, and sodium citrate buffer, incubated at room temperature.

49. The method according to claim 38, further comprising:
filtering the solution;
washing the solution;
digesting the sample; and

purifying the extract through commercially available methods.

50. The method according to claim 49, wherein digesting the sample further comprises lysis and DNase inactivation.

51. The method according to claim 49, wherein digesting the sample further comprises lysis and Endonuclease inactivation.

52. The method according to claim 49, wherein digesting the sample further comprises the steps of:

combining proteinease K, aurintricarboxylic acid, and sodium citrate buffer;

incubating at room temperature; and

eluting the lysate from the filter surface.

53. The method according to claim 38, further comprises applying the solution directly to a biosensor device whereby responsive to the presence of the pathogens in the blood sample, the patient develops pathogenic or native disease state markers which allow for the capture and detection of these markers by the biosensor device.

54. The method according to claim 38, further comprises applying the solution directly to a liquid chromatography mass spectrometry device whereby, responsive to the presence of the pathogens in the blood sample, the patient develops pathogenic or native disease state markers that allow for the detection of mass signatures associated with the structural components of the pathogens using the mass spectrometry device.

55. The method according to claim 38, wherein the buffer comprises Potassium Phosphate, Magnesium Chloride, Sodium Chloride, and Aurintricarboxylic Acid:

56. The method according to claim 55, wherein the buffer further comprises Triton X-100.

57. The method according to claim 55, further comprising the step of storing the enzymes with a trehalose buffer.

58. The method according to claim 57, further comprising the step of combining methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside and Saponin in the trehalose buffer.

59. The method according to claim 58, wherein a concentration of 20-35 mM of methyl 6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside is used.

60. The method according to claim 58, wherein a concentration of 0.05-0.1% Saponin is used.

61. The method according to claim 57, wherein the trehalose storage buffer comprises Potassium Phosphate, Triton X-100, Dithiothreitol, and Trehalose.

62. The method according to claim 61, wherein the trehalose storage buffer comprises 10 mM Potassium Phosphate.

63. The method according to claim 61, wherein the trehalose storage buffer comprises 0.01-0.04% Triton X-100.

64. The method according to claim 61, wherein the trehalose storage buffer comprises 1-5 mM Dithiothreitol.

65. The method according to claim 61, wherein the trehalose storage buffer comprises 0.3-0.5 M Trehalose.

66. A composition comprising urea and EDTA or DTPA or both EDTA and DTPA.

67. The composition according to claim 66, wherein said composition further comprises proteinase K.

68. The composition according to claim 66, wherein said composition further comprises methyl 6-O- (N-heptylcarbamoyl)-α-D-glucopyranoside.

Fig. 1

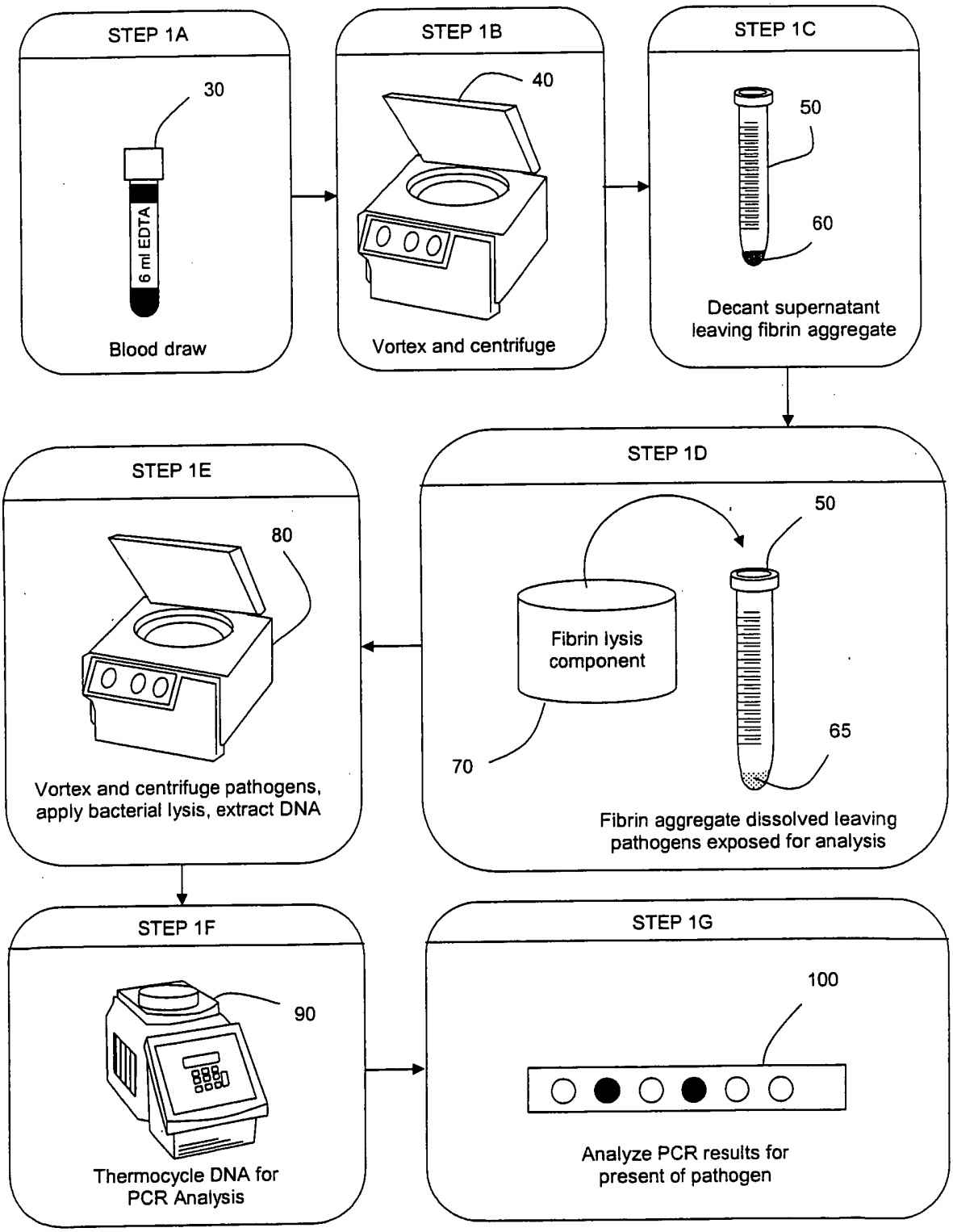
STEP 1A
30
6 ml EDTA
Blood draw
STEP 1B
40
Vortex and centrifuge
STEP 1C
50
60
Decant supernatant leaving fibrin aggregate
STEP 1D
50
Fibrin lysis component
70
65
Fibrin aggregate dissolved leaving pathogens exposed for analysis
STEP 1E
80
Vortex and centrifuge pathogens, apply bacterial lysis, extract DNA
STEP 1F
90
Thermocycle DNA for PCR Analysis
STEP 1G
100
Analyze PCR results for present of pathogen

Fig. 2

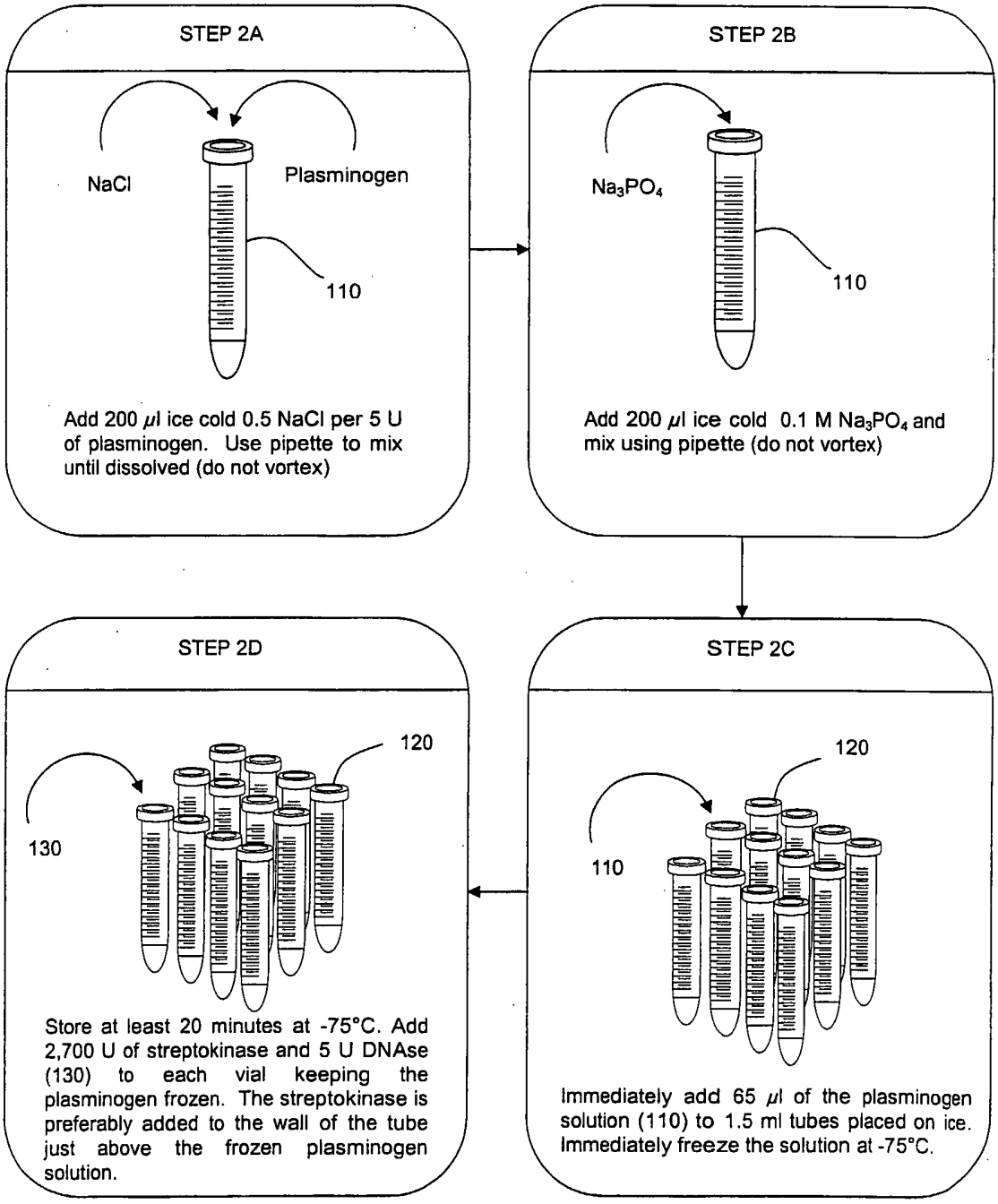
STEP 2A
NaCl
Plasminogen
110
Add 200 µl ice cold 0.5 NaCl per 5 U of plasminogen. Use pipette to mix until dissolved (do not vortex)
STEP 2B
Na_3PO_4
110
Add 200 µl ice cold 0.1 M Na_3PO_4 and mix using pipette (do not vortex)
STEP 2C
120
110
Immediately add 65 µl of the plasminogen solution (110) to 1.5 ml tubes placed on ice. Immediately freeze the solution at -75°C.
STEP 2D
120
130
Store at least 20 minutes at -75°C. Add 2,700 U of streptokinase and 5 U DNAse (130) to each vial keeping the plasminogen frozen. The streptokinase is preferably added to the wall of the tube just above the frozen plasminogen solution.

Fig. 3

***Bacillus anthracis* Blood Protocol Data Set**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Tested 2 Days Post Spiking
M3200253BA1	36.75	37.76	13.75	Spiked Positive
M3200253BA2	36.59	37.86	13.75	Spiked Positive
M3200253BA3	35.97	38.10	13.75	Spiked Positive
M3200253BA4	37.26	39.53	13.75	Spiked Positive
M3200253BA5	35.36	40.11	13.75	Spiked Positive
M3200253BA6	36.35	45.19	13.75	Spiked Positive
M3200253BA7	36.62	38.64	13.75	Spiked Positive
M3200253BA8	37.04	39.51	13.75	Spiked Positive
M320020BA9	0.00	0.00	0.00	Blank
M/3200226BA1	37.16	39.35	1.38	Spiked Positive
M/3200226BA2	36.79	40.28	1.38	Spiked Positive
M/3200226BA3	37.92	39.94	1.38	Spiked Positive
M/3200226BA4	37.49	40.16	1.38	Spiked Positive
M/3200226BA5	39.66	40.26	1.38	Spiked Positive
M/3200226BA6	39.31	41.19	1.38	Spiked Positive
M/3200226BA7	38.48	40.73	1.38	Spiked Positive
M/320020BA8	0.00	0.00	0.00	Blank

Fig. 4

***Bacillus anthracis* Blood Protocol Data Set: Comparison of Blood from Two Different Individuals and Evaluation of Blood Sample Age**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Extracted 84 Days Post Spiking
V210253BA1	37.73	39.81	10.5	Blood Donor #1
V210253BA2	36.74	39.05	10.5	Blood Donor #1
V210253BA3	36.51	37.99	10.5	Blood Donor #1
V210253BA4	38.12	39.79	10.5	Blood Donor #1
V21020BA5	0.00	0.00	0.00	Blank
M210253BA1	37.86	39.81	2.25	Blood Donor #2
M210253BA2	37.84	39.22	2.25	Blood Donor #2
M210253BA3	37.24	38.52	2.25	Blood Donor #2
M210253BA4	38.68	39.33	2.25	Blood Donor #2
M21020BA5	0.00	0.00	0.00	Blank

Fig. 5

***Bacillus anthracis* Blood Protocol Data Set: Evaluation of Blood Protocol by a Department of Health Laboratorian**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type: All Blood Samples Same Batch as in Table 1
M3200256BA1L	38.81	39.93	13.75	Spiked Positive
M3200256BA2L	36.10	39.26	13.75	Spiked Positive
M/3200223BA3L	36.77	38.58	1.38	Spiked Positive
M320020BA4L	0.00	0.00	0.00	Blank

Fig. 6

***Yersinia pestis* Blood Protocol Data Set**

Sample Number	YP 2 Primer / Probes - Crossing Point on Light Cycler	YP 9 Primer / Probes - Crossing Point on Light Cycler	YP12 Primer / Probes - Crossing Point on Light Cycler	YP 16 Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Extracted 2 Days Post Spiking
M3180251EYP1	0.00	0.00	0.00	37.97	12.0	Spiked Positive
M3180251EYP2	0.00	47.01	0.00	0.00	12.0	Spiked Positive
M3180251EYP3	41.56	0.00	0.00	40.29	12.0	Spiked Positive
M3180225EYP4	0.00	0.00	0.00	38.98	24.0	Spiked Positive
M3180225EYP6	40.20	44.01	39.66	37.60	24.0	Spiked Positive
M3180251FYP7	0.00	46.15	0.00	39.79	48.0	Spiked Positive
M3180251FYP8	40.48	43.59	41.70	35.47	48.0	Spiked Positive
M3180251FYP9	40.20	41.88	38.67	34.23	48.0	Spiked Positive
M318020YP10	0.00	0.00	0.00	0.00	0.00	Blank

Fig. 7

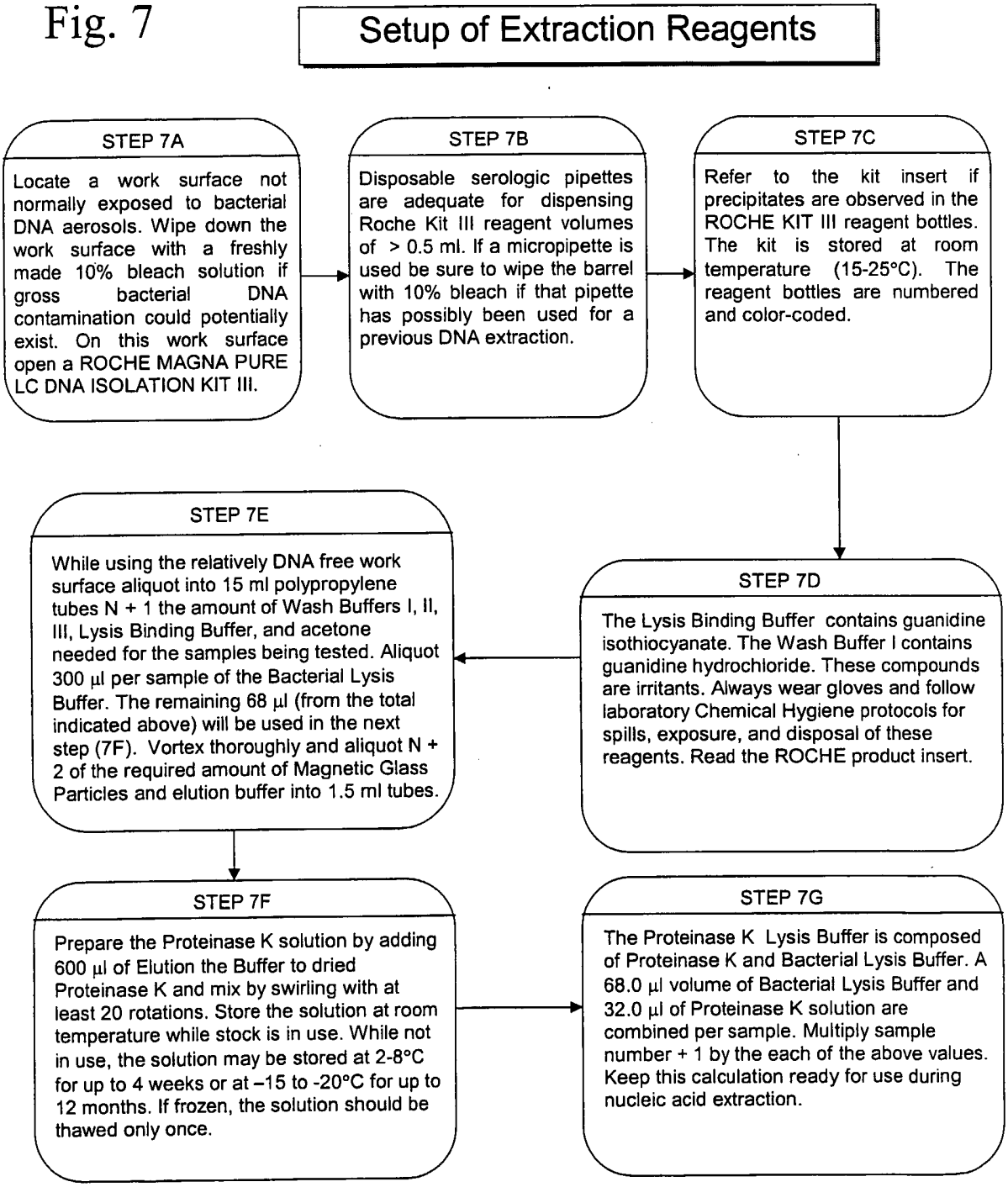
Setup of Extraction Reagents
STEP 7A
Locate a work surface not normally exposed to bacterial DNA aerosols. Wipe down the work surface with a freshly made 10% bleach solution if gross bacterial DNA contamination could potentially exist. On this work surface open a ROCHE MAGNA PURE LC DNA ISOLATION KIT III.
STEP 7B
Disposable serologic pipettes are adequate for dispensing Roche Kit III reagent volumes of > 0.5 ml. If a micropipette is used be sure to wipe the barrel with 10% bleach if that pipette has possibly been used for a previous DNA extraction.
STEP 7C
Refer to the kit insert if precipitates are observed in the ROCHE KIT III reagent bottles. The kit is stored at room temperature (15-25°C). The reagent bottles are numbered and color-coded.
STEP 7D
The Lysis Binding Buffer contains guanidine isothiocyanate. The Wash Buffer I contains guanidine hydrochloride. These compounds are irritants. Always wear gloves and follow laboratory Chemical Hygiene protocols for spills, exposure, and disposal of these reagents. Read the ROCHE product insert.
STEP 7E
While using the relatively DNA free work surface aliquot into 15 ml polypropylene tubes N + 1 the amount of Wash Buffers I, II, III, Lysis Binding Buffer, and acetone needed for the samples being tested. Aliquot 300 µl per sample of the Bacterial Lysis Buffer. The remaining 68 µl (from the total indicated above) will be used in the next step (7F). Vortex thoroughly and aliquot N + 2 of the required amount of Magnetic Glass Particles and elution buffer into 1.5 ml tubes.
STEP 7F
Prepare the Proteinase K solution by adding 600 µl of Elution the Buffer to dried Proteinase K and mix by swirling with at least 20 rotations. Store the solution at room temperature while stock is in use. While not in use, the solution may be stored at 2-8°C for up to 4 weeks or at –15 to -20°C for up to 12 months. If frozen, the solution should be thawed only once.
STEP 7G
The Proteinase K Lysis Buffer is composed of Proteinase K and Bacterial Lysis Buffer. A 68.0 µl volume of Bacterial Lysis Buffer and 32.0 µl of Proteinase K solution are combined per sample. Multiply sample number + 1 by the each of the above values. Keep this calculation ready for use during nucleic acid extraction.

Fig. 8

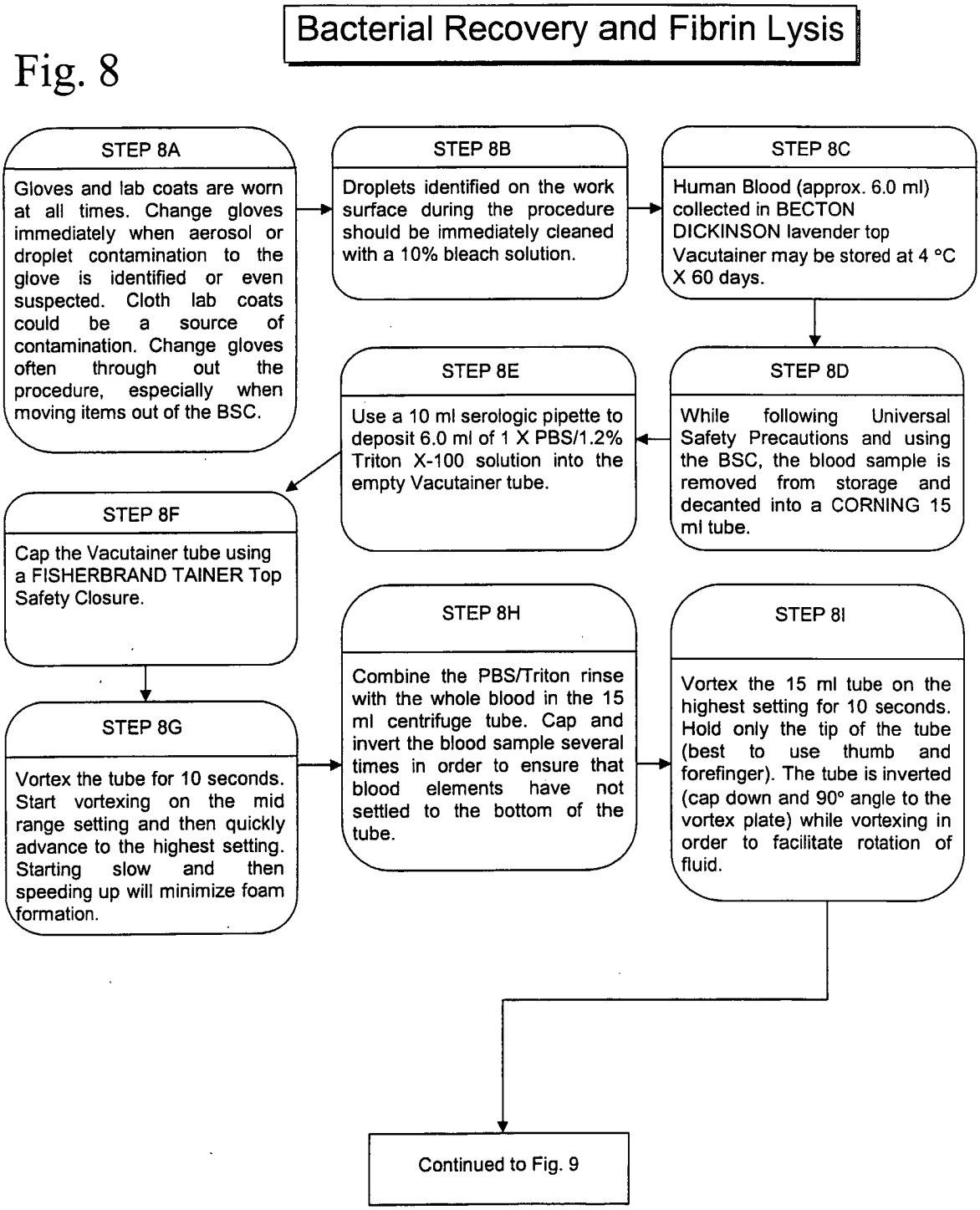
Bacterial Recovery and Fibrin Lysis
STEP 8A
Gloves and lab coats are worn at all times. Change gloves immediately when aerosol or droplet contamination to the glove is identified or even suspected. Cloth lab coats could be a source of contamination. Change gloves often through out the procedure, especially when moving items out of the BSC.
STEP 8B
Droplets identified on the work surface during the procedure should be immediately cleaned with a 10% bleach solution.
STEP 8C
Human Blood (approx. 6.0 ml) collected in BECTON DICKINSON lavender top Vacutainer may be stored at 4 °C X 60 days.
STEP 8D
While following Universal Safety Precautions and using the BSC, the blood sample is removed from storage and decanted into a CORNING 15 ml tube.
STEP 8E
Use a 10 ml serologic pipette to deposit 6.0 ml of 1 X PBS/1.2% Triton X-100 solution into the empty Vacutainer tube.
STEP 8F
Cap the Vacutainer tube using a FISHERBRAND TAINER Top Safety Closure.
STEP 8G
Vortex the tube for 10 seconds. Start vortexing on the mid range setting and then quickly advance to the highest setting. Starting slow and then speeding up will minimize foam formation.
STEP 8H
Combine the PBS/Triton rinse with the whole blood in the 15 ml centrifuge tube. Cap and invert the blood sample several times in order to ensure that blood elements have not settled to the bottom of the tube.
STEP 8I
Vortex the 15 ml tube on the highest setting for 10 seconds. Hold only the tip of the tube (best to use thumb and forefinger). The tube is inverted (cap down and 90° angle to the vortex plate) while vortexing in order to facilitate rotation of fluid.
Continued to Fig. 9

Fig. 9

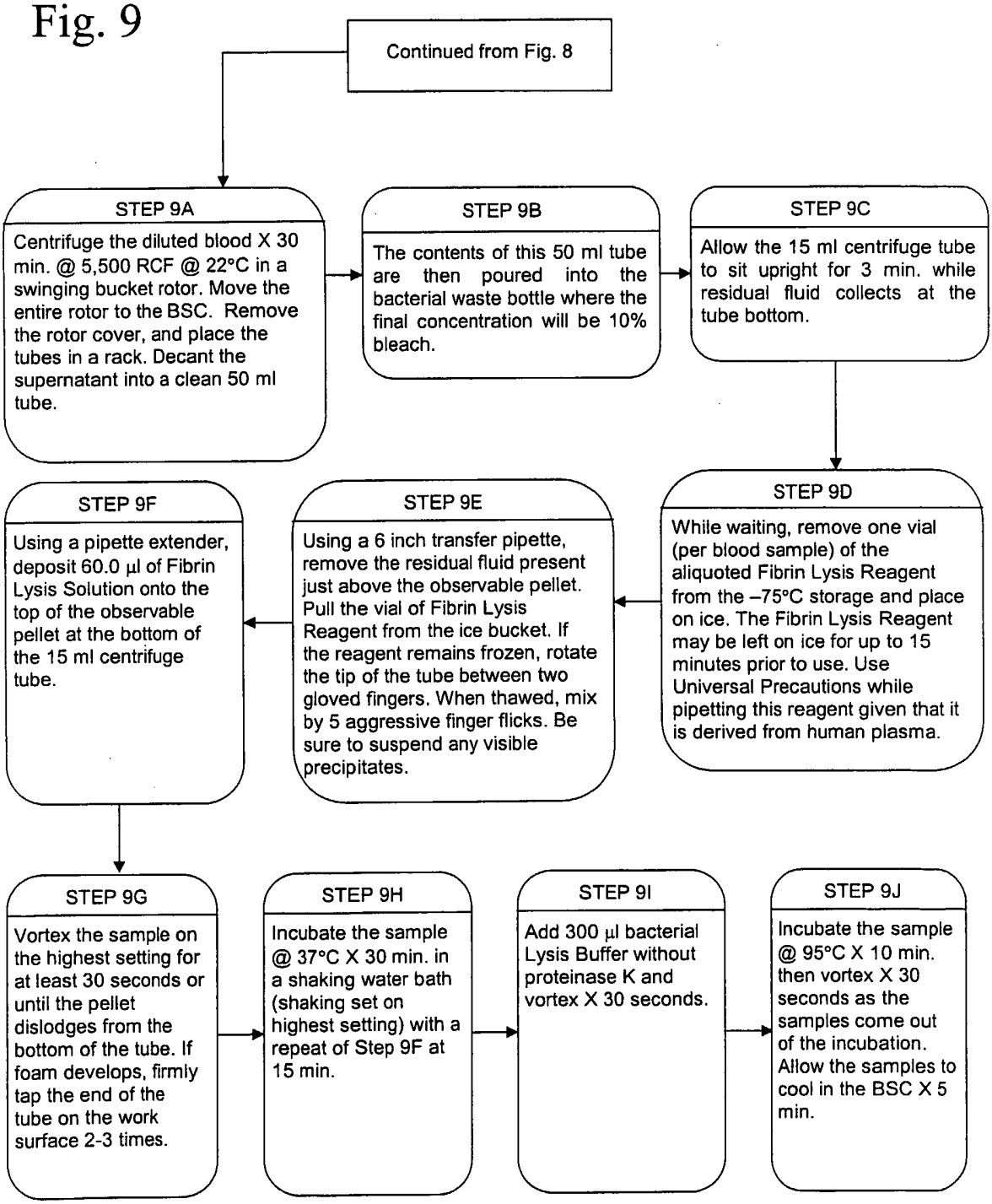
Continued from Fig. 8
STEP 9A
Centrifuge the diluted blood X 30 min. @ 5,500 RCF @ 22°C in a swinging bucket rotor. Move the entire rotor to the BSC. Remove the rotor cover, and place the tubes in a rack. Decant the supernatant into a clean 50 ml tube.
STEP 9B
The contents of this 50 ml tube are then poured into the bacterial waste bottle where the final concentration will be 10% bleach.
STEP 9C
Allow the 15 ml centrifuge tube to sit upright for 3 min. while residual fluid collects at the tube bottom.
STEP 9D
While waiting, remove one vial (per blood sample) of the aliquoted Fibrin Lysis Reagent from the –75°C storage and place on ice. The Fibrin Lysis Reagent may be left on ice for up to 15 minutes prior to use. Use Universal Precautions while pipetting this reagent given that it is derived from human plasma.
STEP 9E
Using a 6 inch transfer pipette, remove the residual fluid present just above the observable pellet. Pull the vial of Fibrin Lysis Reagent from the ice bucket. If the reagent remains frozen, rotate the tip of the tube between two gloved fingers. When thawed, mix by 5 aggressive finger flicks. Be sure to suspend any visible precipitates.
STEP 9F
Using a pipette extender, deposit 60.0 µl of Fibrin Lysis Solution onto the top of the observable pellet at the bottom of the 15 ml centrifuge tube.
STEP 9G
Vortex the sample on the highest setting for at least 30 seconds or until the pellet dislodges from the bottom of the tube. If foam develops, firmly tap the end of the tube on the work surface 2-3 times.
STEP 9H
Incubate the sample @ 37°C X 30 min. in a shaking water bath (shaking set on highest setting) with a repeat of Step 9F at 15 min.
STEP 9I
Add 300 µl bacterial Lysis Buffer without proteinase K and vortex X 30 seconds.
STEP 9J
Incubate the sample @ 95°C X 10 min. then vortex X 30 seconds as the samples come out of the incubation. Allow the samples to cool in the BSC X 5 min.

Fig. 10

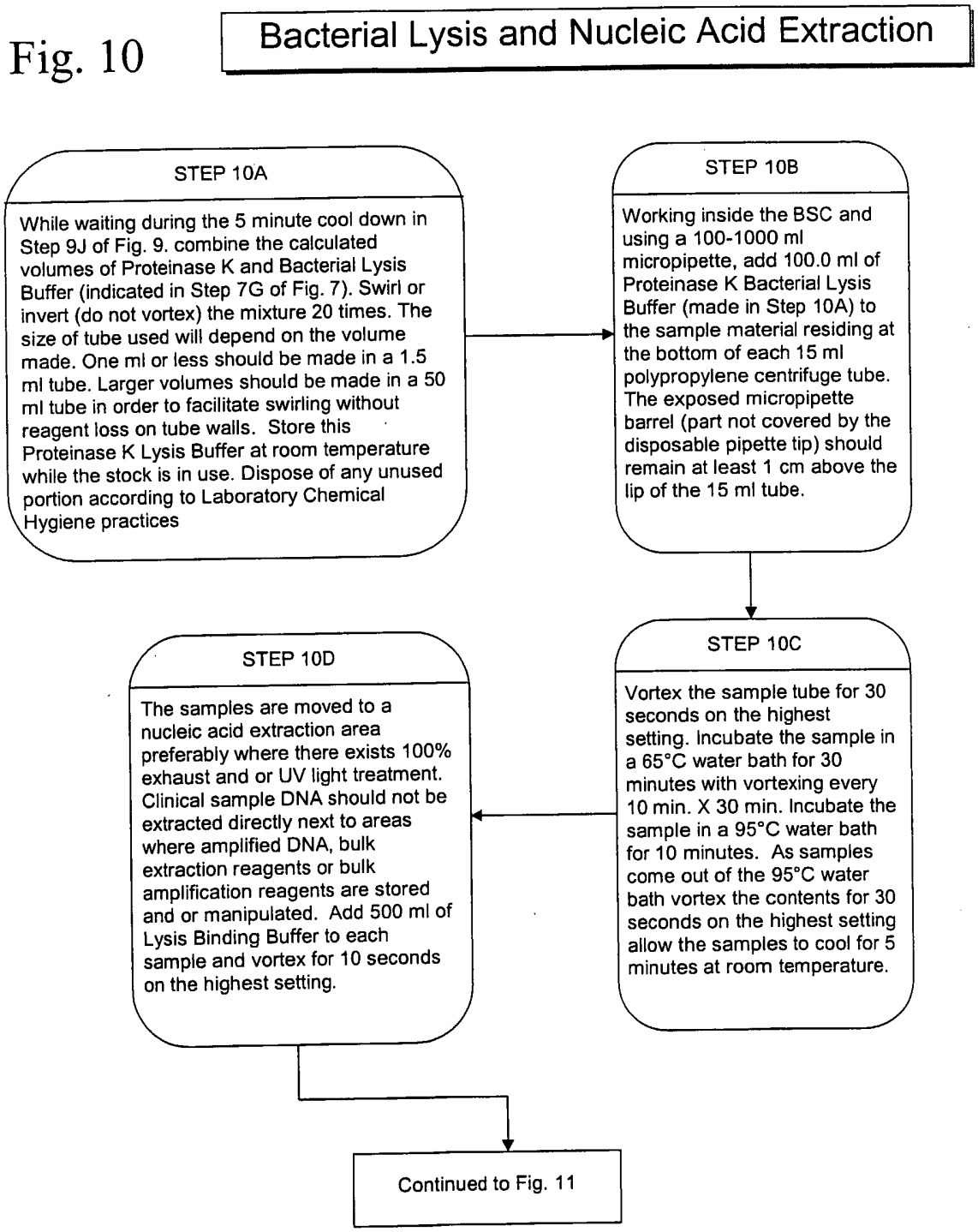
Bacterial Lysis and Nucleic Acid Extraction
STEP 10A
While waiting during the 5 minute cool down in Step 9J of Fig. 9. combine the calculated volumes of Proteinase K and Bacterial Lysis Buffer (indicated in Step 7G of Fig. 7). Swirl or invert (do not vortex) the mixture 20 times. The size of tube used will depend on the volume made. One ml or less should be made in a 1.5 ml tube. Larger volumes should be made in a 50 ml tube in order to facilitate swirling without reagent loss on tube walls. Store this Proteinase K Lysis Buffer at room temperature while the stock is in use. Dispose of any unused portion according to Laboratory Chemical Hygiene practices
STEP 10B
Working inside the BSC and using a 100-1000 ml micropipette, add 100.0 ml of Proteinase K Bacterial Lysis Buffer (made in Step 10A) to the sample material residing at the bottom of each 15 ml polypropylene centrifuge tube. The exposed micropipette barrel (part not covered by the disposable pipette tip) should remain at least 1 cm above the lip of the 15 ml tube.
STEP 10C
Vortex the sample tube for 30 seconds on the highest setting. Incubate the sample in a 65°C water bath for 30 minutes with vortexing every 10 min. X 30 min. Incubate the sample in a 95°C water bath for 10 minutes. As samples come out of the 95°C water bath vortex the contents for 30 seconds on the highest setting allow the samples to cool for 5 minutes at room temperature.
STEP 10D
The samples are moved to a nucleic acid extraction area preferably where there exists 100% exhaust and or UV light treatment. Clinical sample DNA should not be extracted directly next to areas where amplified DNA, bulk extraction reagents or bulk amplification reagents are stored and or manipulated. Add 500 ml of Lysis Binding Buffer to each sample and vortex for 10 seconds on the highest setting.
Continued to Fig. 11

Fig. 11

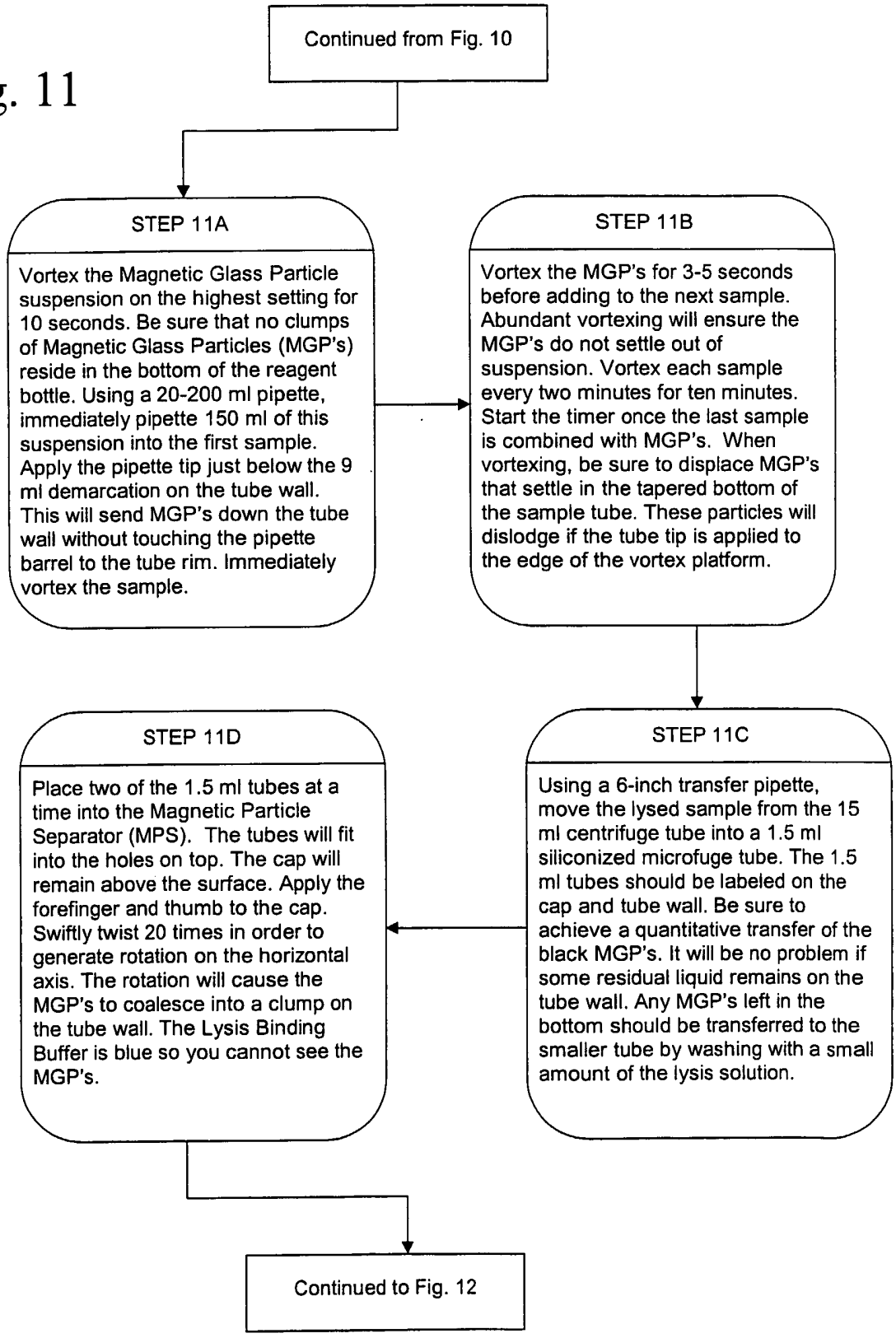
Continued from Fig. 10
STEP 11A
Vortex the Magnetic Glass Particle suspension on the highest setting for 10 seconds. Be sure that no clumps of Magnetic Glass Particles (MGP's) reside in the bottom of the reagent bottle. Using a 20-200 ml pipette, immediately pipette 150 ml of this suspension into the first sample. Apply the pipette tip just below the 9 ml demarcation on the tube wall. This will send MGP's down the tube wall without touching the pipette barrel to the tube rim. Immediately vortex the sample.
STEP 11B
Vortex the MGP's for 3-5 seconds before adding to the next sample. Abundant vortexing will ensure the MGP's do not settle out of suspension. Vortex each sample every two minutes for ten minutes. Start the timer once the last sample is combined with MGP's. When vortexing, be sure to displace MGP's that settle in the tapered bottom of the sample tube. These particles will dislodge if the tube tip is applied to the edge of the vortex platform.
STEP 11C
Using a 6-inch transfer pipette, move the lysed sample from the 15 ml centrifuge tube into a 1.5 ml siliconized microfuge tube. The 1.5 ml tubes should be labeled on the cap and tube wall. Be sure to achieve a quantitative transfer of the black MGP's. It will be no problem if some residual liquid remains on the tube wall. Any MGP's left in the bottom should be transferred to the smaller tube by washing with a small amount of the lysis solution.
STEP 11D
Place two of the 1.5 ml tubes at a time into the Magnetic Particle Separator (MPS). The tubes will fit into the holes on top. The cap will remain above the surface. Apply the forefinger and thumb to the cap. Swiftly twist 20 times in order to generate rotation on the horizontal axis. The rotation will cause the MGP's to coalesce into a clump on the tube wall. The Lysis Binding Buffer is blue so you cannot see the MGP's.
Continued to Fig. 12

Fig. 12

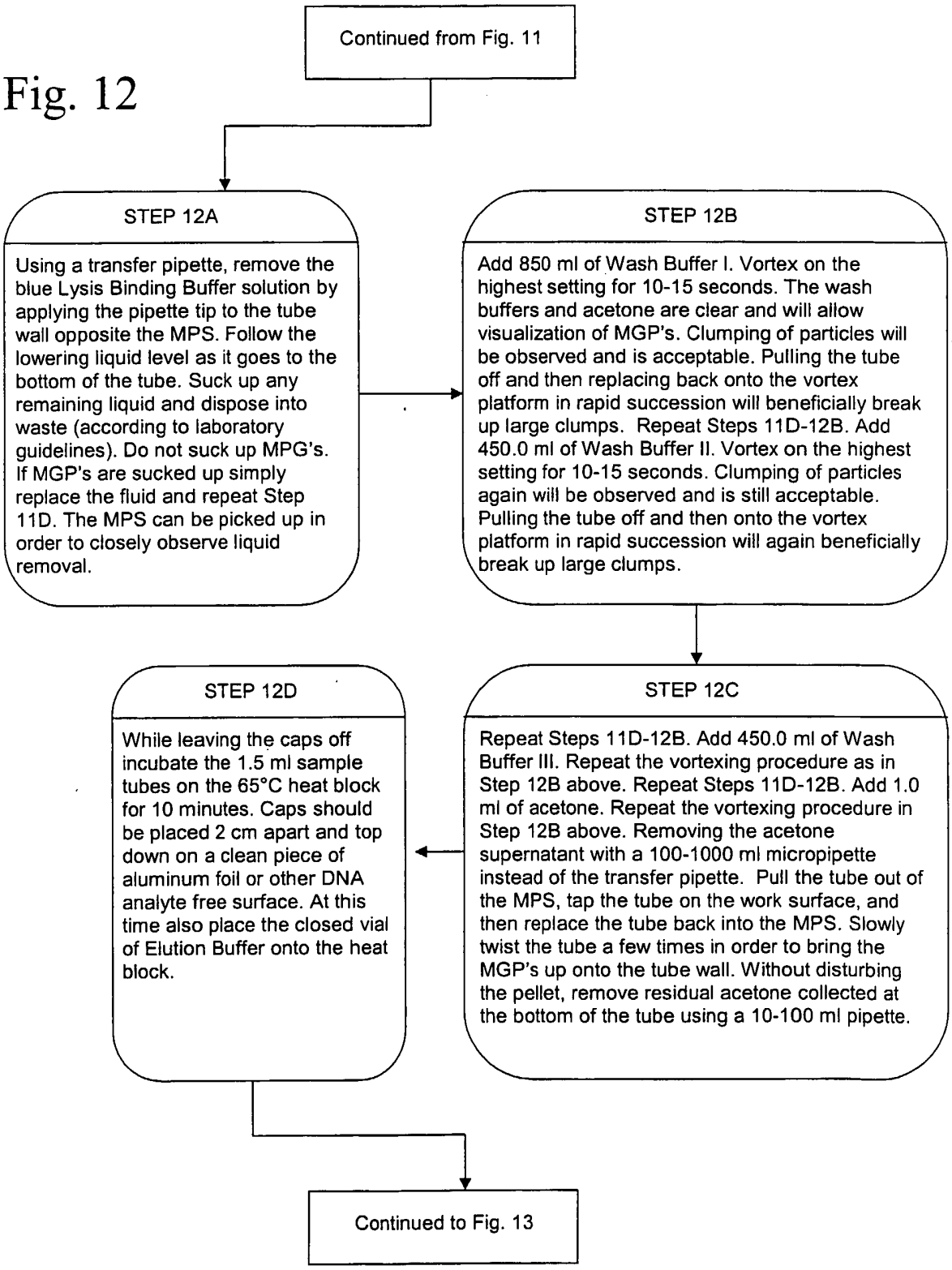
Continued from Fig. 11
STEP 12A
Using a transfer pipette, remove the blue Lysis Binding Buffer solution by applying the pipette tip to the tube wall opposite the MPS. Follow the lowering liquid level as it goes to the bottom of the tube. Suck up any remaining liquid and dispose into waste (according to laboratory guidelines). Do not suck up MPG's. If MGP's are sucked up simply replace the fluid and repeat Step 11D. The MPS can be picked up in order to closely observe liquid removal.
STEP 12B
Add 850 ml of Wash Buffer I. Vortex on the highest setting for 10-15 seconds. The wash buffers and acetone are clear and will allow visualization of MGP's. Clumping of particles will be observed and is acceptable. Pulling the tube off and then replacing back onto the vortex platform in rapid succession will beneficially break up large clumps. Repeat Steps 11D-12B. Add 450.0 ml of Wash Buffer II. Vortex on the highest setting for 10-15 seconds. Clumping of particles again will be observed and is still acceptable. Pulling the tube off and then onto the vortex platform in rapid succession will again beneficially break up large clumps.
STEP 12C
Repeat Steps 11D-12B. Add 450.0 ml of Wash Buffer III. Repeat the vortexing procedure as in Step 12B above. Repeat Steps 11D-12B. Add 1.0 ml of acetone. Repeat the vortexing procedure in Step 12B above. Removing the acetone supernatant with a 100-1000 ml micropipette instead of the transfer pipette. Pull the tube out of the MPS, tap the tube on the work surface, and then replace the tube back into the MPS. Slowly twist the tube a few times in order to bring the MGP's up onto the tube wall. Without disturbing the pellet, remove residual acetone collected at the bottom of the tube using a 10-100 ml pipette.
STEP 12D
While leaving the caps off incubate the 1.5 ml sample tubes on the 65°C heat block for 10 minutes. Caps should be placed 2 cm apart and top down on a clean piece of aluminum foil or other DNA analyte free surface. At this time also place the closed vial of Elution Buffer onto the heat block.
Continued to Fig. 13

Fig. 13

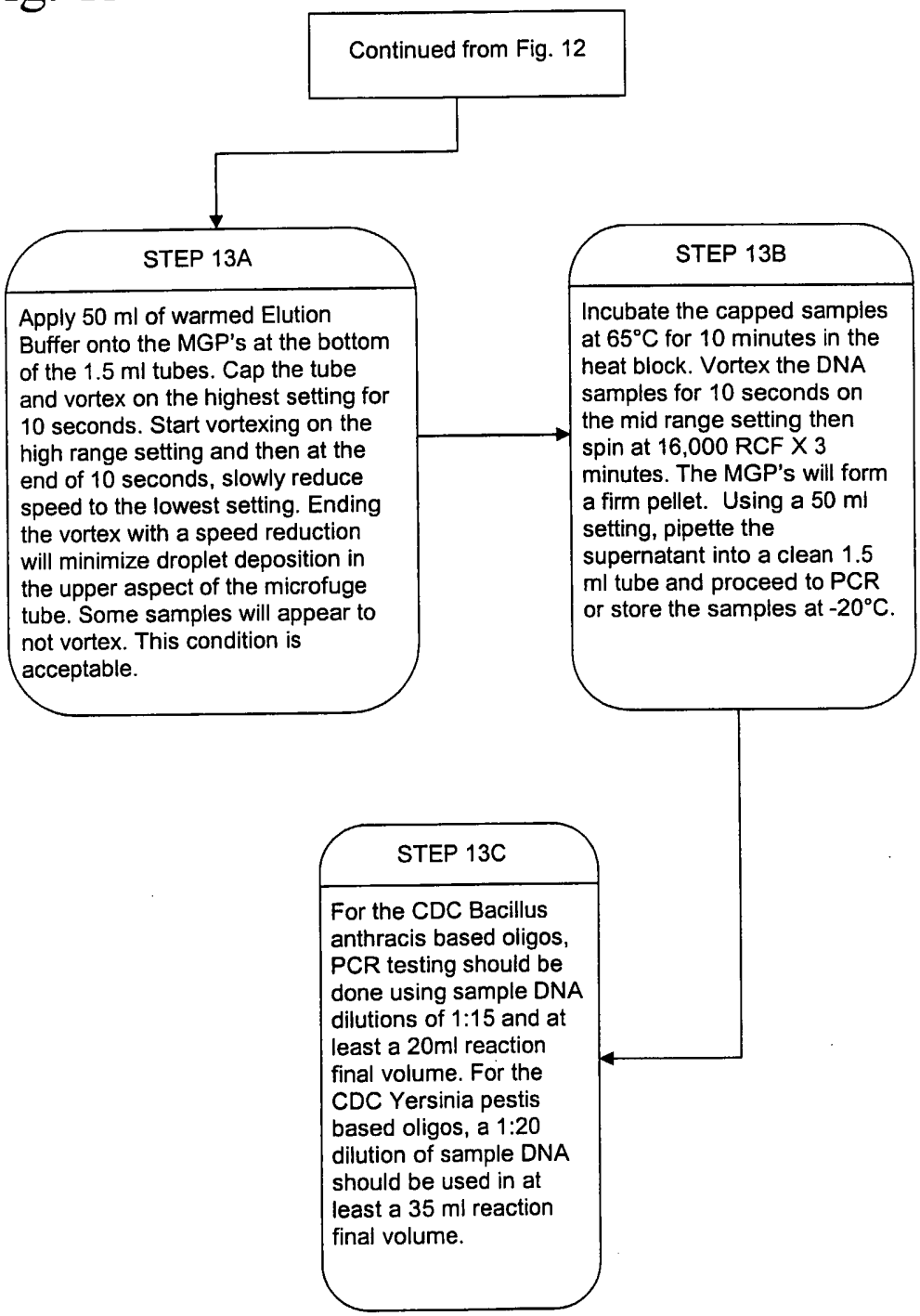
Continued from Fig. 12
STEP 13A
Apply 50 ml of warmed Elution Buffer onto the MGP's at the bottom of the 1.5 ml tubes. Cap the tube and vortex on the highest setting for 10 seconds. Start vortexing on the high range setting and then at the end of 10 seconds, slowly reduce speed to the lowest setting. Ending the vortex with a speed reduction will minimize droplet deposition in the upper aspect of the microfuge tube. Some samples will appear to not vortex. This condition is acceptable.
STEP 13B
Incubate the capped samples at 65°C for 10 minutes in the heat block. Vortex the DNA samples for 10 seconds on the mid range setting then spin at 16,000 RCF X 3 minutes. The MGP's will form a firm pellet. Using a 50 ml setting, pipette the supernatant into a clean 1.5 ml tube and proceed to PCR or store the samples at -20°C.
STEP 13C
For the CDC Bacillus anthracis based oligos, PCR testing should be done using sample DNA dilutions of 1:15 and at least a 20ml reaction final volume. For the CDC Yersinia pestis based oligos, a 1:20 dilution of sample DNA should be used in at least a 35 ml reaction final volume.

Fig. 14a

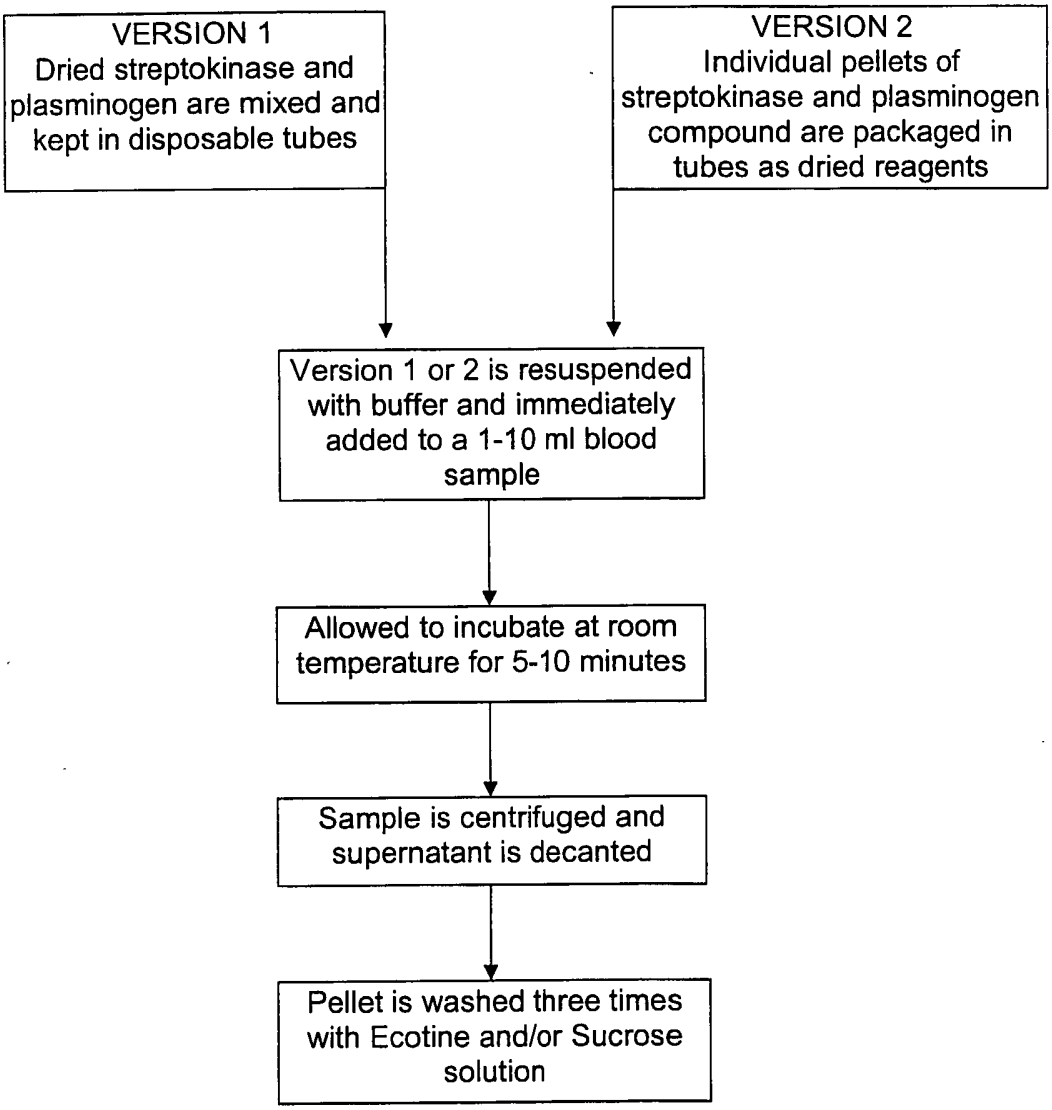
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Sample is centrifuged and supernatant is decanted
Pellet is washed three times with Ecotine and/or Sucrose solution

Fig. 14b

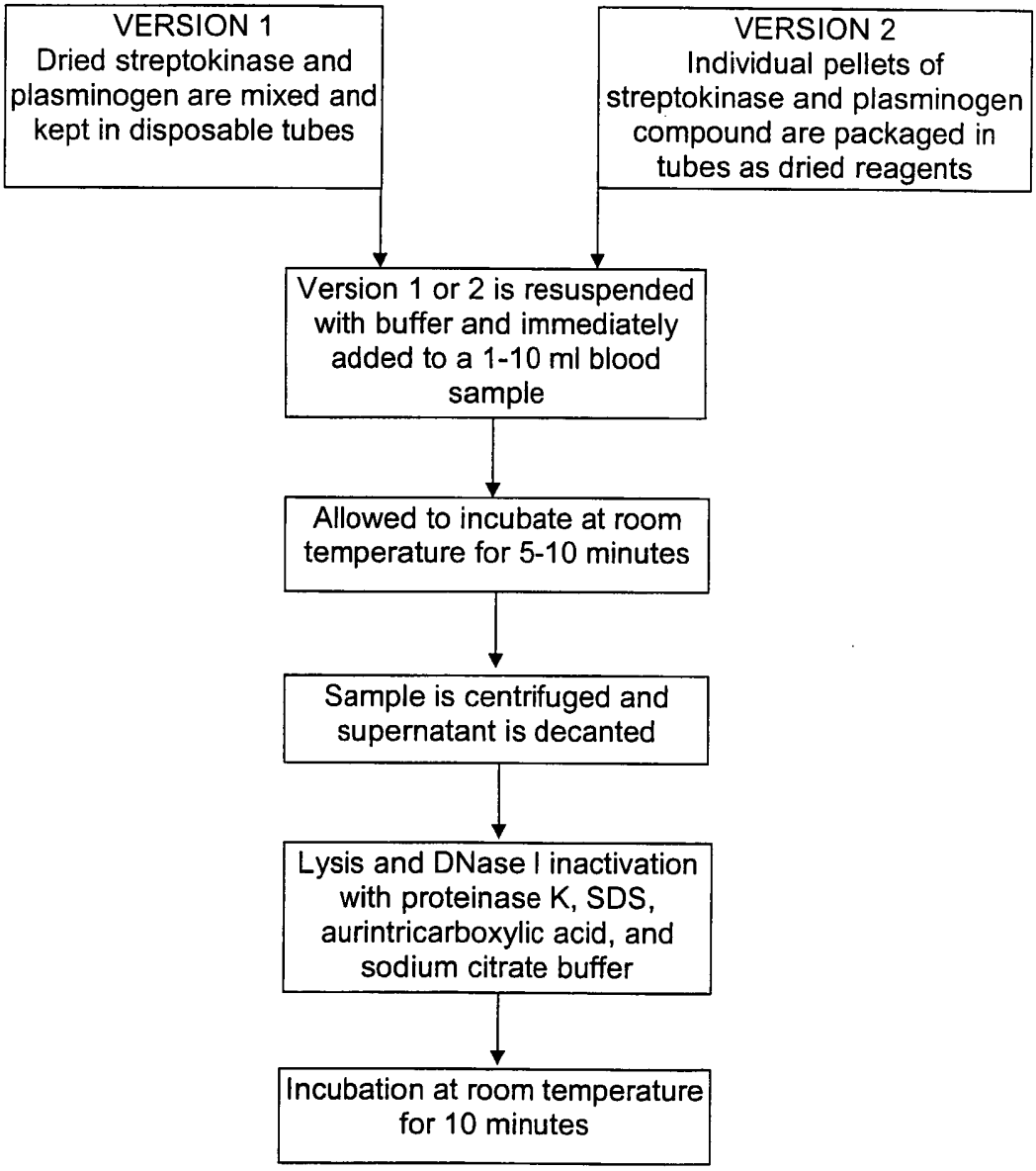
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Sample is centrifuged and supernatant is decanted
Lysis and DNase I inactivation with proteinase K, SDS, aurintricarboxylic acid, and sodium citrate buffer
Incubation at room temperature for 10 minutes

Fig. 15

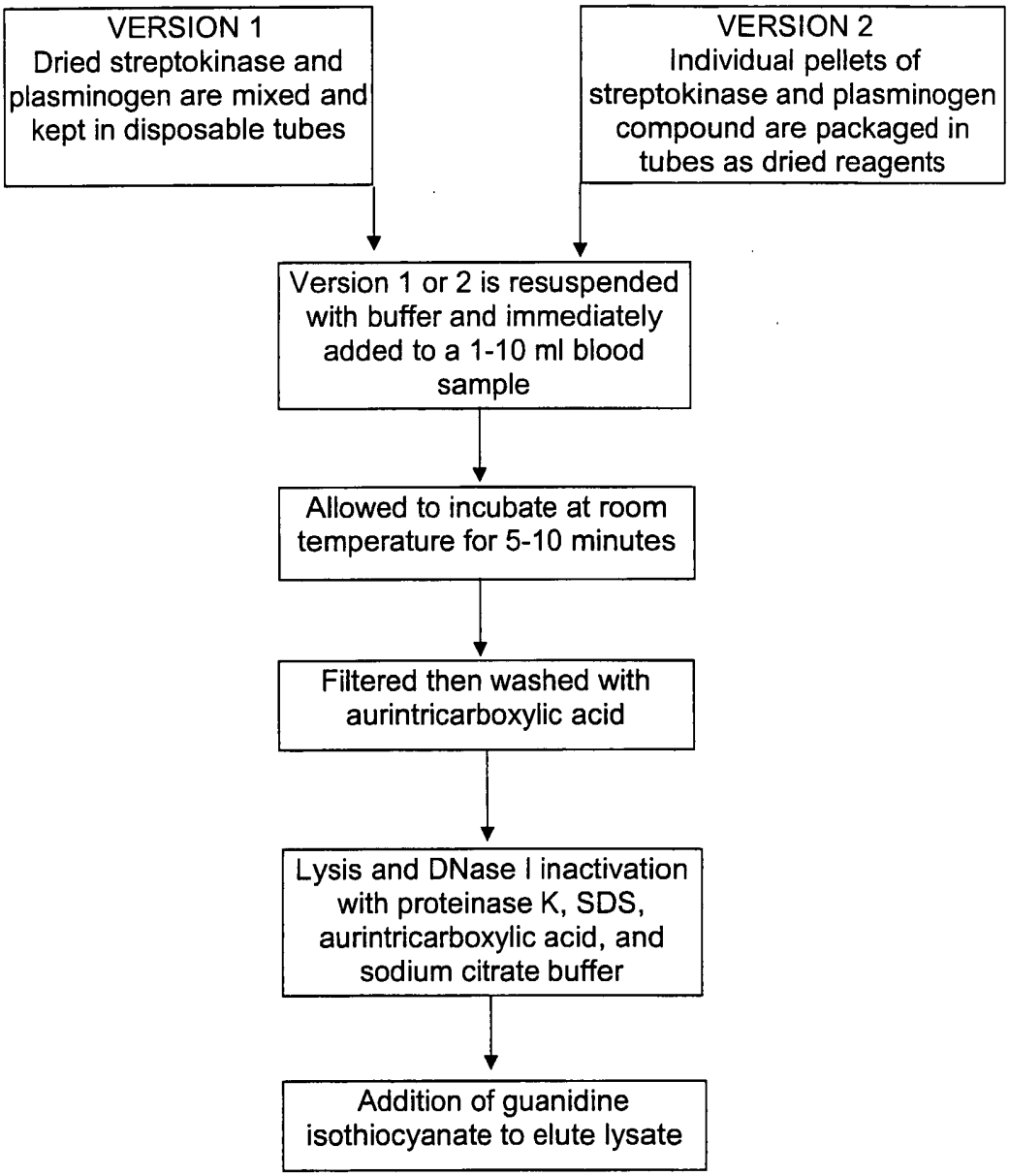
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Filtered then washed with aurintricarboxylic acid
Lysis and DNase I inactivation with proteinase K, SDS, aurintricarboxylic acid, and sodium citrate buffer
Addition of guanidine isothiocyanate to elute lysate

Fig. 16a

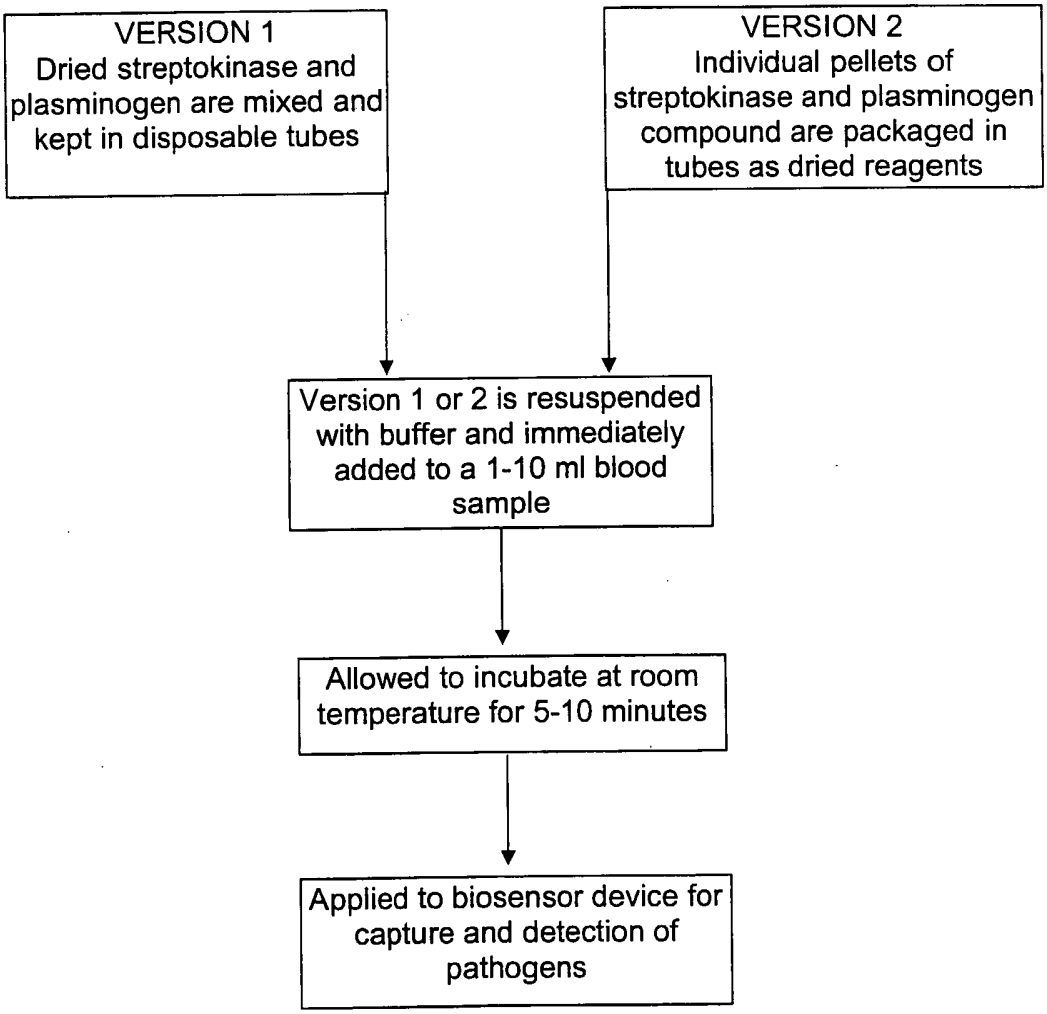
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Applied to biosensor device for capture and detection of pathogens

Fig. 16b

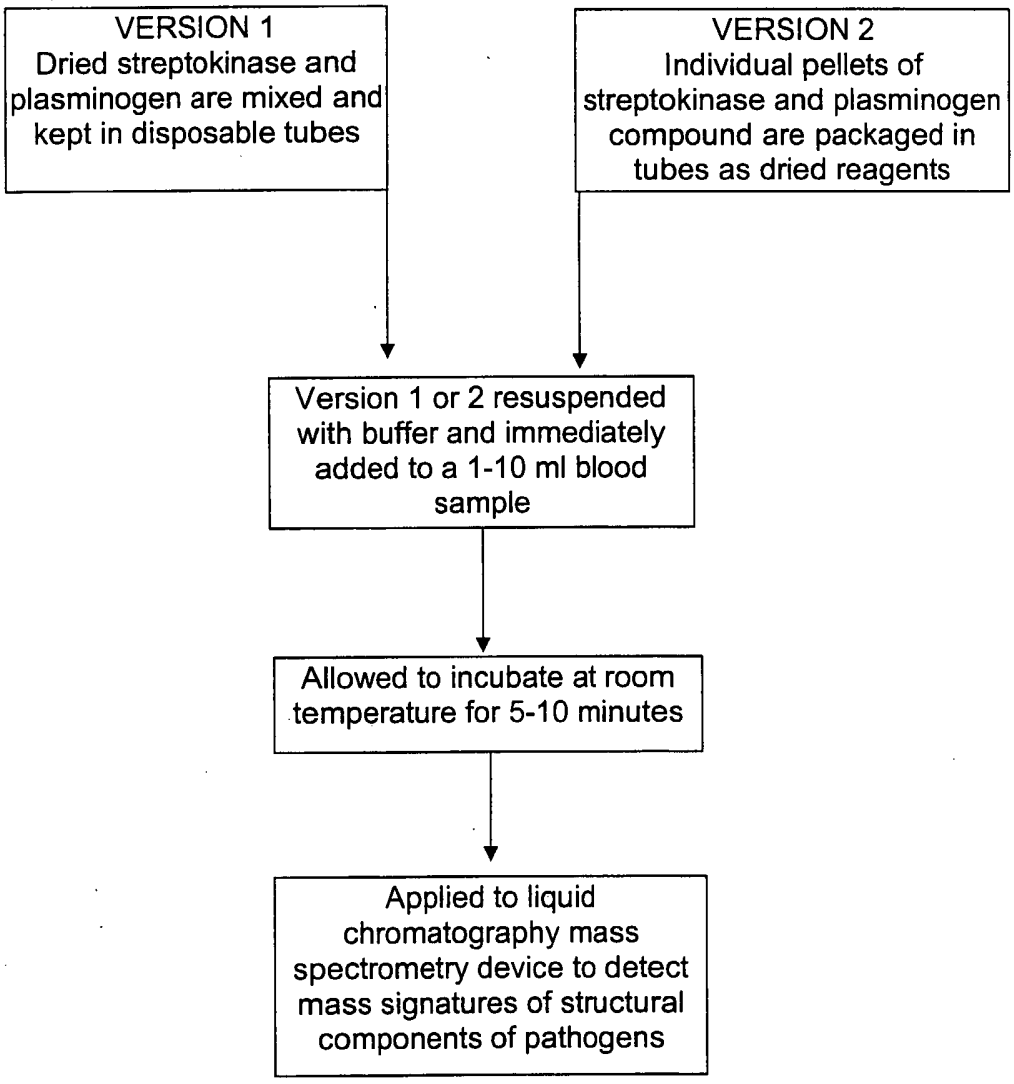
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Applied to liquid chromatography mass spectrometry device to detect mass signatures of structural components of pathogens

Fig. 17

Noise band crossing points for blood samples spiked with *B. anthracis* and processed with plasminogen, streptokinase, phospholipase A_2, DNase I, and lipase with centrifugation or filtration

	Centrifugation					Filtration				
Amount *B. anthracis* Seeded (cfu)	Noise Band Crossing Points			Mean	Std. Dev.	Noise Band Crossing Points			Mean	Std. Dev.
≤ 0.01										
≤ 0.01										
≤ 1.0						40.33	39.89		40.11	
≤ 1.0							37.79		37.79	
≤ 2.0						40.36		37.69	39.03	
≤ 2.0	41.93		40.31	41.12						
≤ 5.0			40.47	40.47		37.90	37.70	37.79	37.80	0.10
≤ 5.0	38.11		40.36	39.24		36.45	36.09	36.81	36.45	0.36
≤ 50.0	37.53	36.24	37.90	37.22	0.87	35.75	34.12	34.98	34.95	0.82
≤ 50.0	36.45	38.15	38.49	37.70	1.09	35.24	34.18	34.68	34.70	0.53

Fig. 18

Sedimentation and solublization of tissue aggregates from 6 ml blood samples exposed to various detergent and enzyme treatments

	Enzyme treatments in a PBS/Triton X-100 buffer						
	Triton X-100 in PBS	Pl.[c] 1U	Ph.[b]	Pl.[c] 1U Ph.[b]	Dn.[a] 1mg	Dn.[a] 1 mg Ph.[b]	Dn.[a] 1 mg Pl.[c] 1U Ph.[b]
% Observable pelleted tissue aggregate post centrifugation	100	100	100	100	90	10	10
Time (min) to solubilization of visible tissue aggregate in BLB[d]	> 360	> 60	> 60	> 60	< 10	< 0.5	< 0.5

[a] DNase I from the Roche MagNa Pure LC DNA Kit III
[b] Phospholipase A_2
[c] Plasminogen and 10K U streptokinase
[d] Bacterial Lysis Buffer from the Roche MagNa Pure LC DNA Kit III

Fig. 19

Filtration characteristics of 6 ml blood samples exposed to various detergent and enzyme treatments

		Enzyme treatments in a PBS/Triton X-100 buffer					
	Triton X-100 in PBS	Dn.[a] 1mg	Dn.[a] 1 mg Ph.[b]	Pl.[c] 5U	Pl.[c] 5U Dn.[a] 1mg Ph.[b]	Pl.[c] 5U Dn.[a] 0.2mg Ph.[b]	Pl.[c] 10U Dn.[a] 0.2mg Ph.[b]
Not filterable	+	+	+				
Filterable with observable tissue aggregates				+		+	
Filterable with out observable aggregates					+		+

[a] DNase I from the Roche MagNa Pure LC DNA Kit III

[b] Phospholipase A_2

[c] Plasminogen converted to plasmin with 10K U streptokinase