METHODS AND REAGENTS FOR REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Abstract: The present invention provides a method of amplifying an RNA molecule in a biological sample by reverse transcription PCR (RT-PCR), wherein the RT-PCR is carried out in a solution comprising a) a polar aprotic solvent; b) a serum albumin; and optionally c) a non-ionic surfactant and/or a betaine.
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METHODS AND REAGENTS FOR REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

FIELD OF THE INVENTION

The present invention provides methods for reverse-transcription polymerase chain reaction. The present invention also provides compositions for use in reverse-transcription polymerase chain reactions.

BACKGROUND TO THE INVENTION

The reverse transcription polymerase chain reaction (RT-PCR) is a widely used method for amplifying RNA. In the RT-PCR reaction, a DNA molecule (termed a complementary DNA molecule, which can be abbreviated to a "cDNA molecule") is generated from a single stranded RNA template using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PGR. The cDNA sequence may be generated from the full length of the mRNA sequence or a portion of the mRNA sequence.

RT-PCR has many applications, including the diagnosis of microorganisms such as RNA-viruses, viral load monitoring during drug treatment, identification of genetic diseases, gene expression analysis and the typing of genetically modified organisms etc. Traditional RT-PCR based assays rely on RNA extraction prior to amplification, thus removing any potential RT-PCR inhibitors present in the biological material. This extraction or purification step is however, associated with several disadvantages. For example: i) it can cause the cross contamination of samples, causing the generation of false positives, which is especially critical during high throughput RNA-based screening assays such as those employed to monitor the presence of RNA-based viruses such as HIV and Ebola in blood; ii) it can be a source of infection unless the operators are following specific laboratory safety or containment measures; iii) it can be labour intensive; iv) it can be costly in terms of both material and time; and v) it does not always provide a robust and reliable method for the removal of some RT-PCR inhibitors.

One of the major challenges with developing novel methodologies for carrying out PCR directly from crude samples is the presence of compounds that inhibit the amplification of nucleic acids, such as haem in blood. One approach investigators have used to attempt to overcome this challenge is to replace the wild-type DNA polymerases with a
genetically engineered enzyme that is more resistant to several common inhibitors (Al-
products consist of mutated Taq DNA polymerases that are suitable for direct PCR,
there are currently no alternatives for the direct RT-PCR amplification of RNA from
crude biological samples such as blood and tissue. Inhibitor-resistant reverse
transcriptase (RT) enzymes are not readily available. The development of a direct RT-
PCR workflow and its incorporation into a freeze-dried format such as the GE
Healthcare "Ready to Go" formulation will provide major benefits e.g. time saving and
simplified workflows. Such reagents will also deliver ambient storage and transportation
benefits.

SUMMARY OF THE INVENTION

According to a first aspect of the present invention, there is provided a method of
amplifying an RNA molecule in a biological sample by reverse transcription PCR (RT-
PCR), wherein the RT-PCR is carried out in a solution comprising: a polar aprotic
solvent; a serum albumin; and optionally a non-ionic surfactant and/or a betaine.

In one embodiment, the solution comprises a polar aprotic solvent; a serum albumin; and
a betaine.

In a further embodiment, the solution comprises: a polar aprotic solvent; a serum
albumin; a non-ionic surfactant and a betaine.

In a further embodiment, the non-ionic surfactant is Triton X-100, Brij 56 or Brij 58.

In a further embodiment, the polar aprotic solvent is DMSO (dimethyl sulfoxide).

In a further embodiment, the betaine is \( N,N,N \)-trimethylglycine.

In a further embodiment, the solution comprises: (a) DMSO at a concentration in the
range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to 1.2% (w/v); Triton
X-100 at a concentration in the range 0.05 to 1% (v/v); and \( N,N,N \)-trimethylglycine at a
concentration in the range 0.05 to 2M; or (b) DMSO at a concentration in the range 0.05
to 10% (v/v), BSA at a concentration in the range 0.05 to 1.2% (w/v); or (c) DMSO at a
concentration in the range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to
1.2% (w/v); and \( N,N,N \)-trimethylglycine at a concentration in the range 0.05 to 2M.
In a further embodiment, the solution further comprises: a reverse transcriptase; a DNA polymerase; a deoxyribonucleotide triphosphate (dNTP); and at least one primer.

In a further embodiment, the biological sample has not undergone treatment with a DNase prior to the RT-PCR reaction. In a further embodiment, the biological sample has not undergone an RNA purification step prior to the RT-PCR reaction.

In a further embodiment, the method comprises subjecting the biological sample to a lysis step and a detergent neutralization step prior to contacting the biological sample with the solution or prior to the RT-PCR reaction. In a further embodiment, the lysis step is performed by contacting the biological sample with a detergent, preferably sodium dodecyl sulphate (SDS), and the detergent neutralization step is performed by contacting the sample with a cyclodextran.

In a further embodiment, the biological sample comprises a cellular sample selected from the group consisting of blood, serum, semen, cerebral spinal fluid, synovial fluid, lymphatic fluid, saliva, buccal, cervical cell, vaginal cell, urine, faeces, hair, skin, muscle, and cells grown in culture. In a further embodiment, the biological sample is derived from a virus, a eukaryotic organism or a prokaryotic organism. In a further embodiment, the biological sample is a blood sample, optionally where the blood has been treated with an anti-coagulant.

In a further embodiment, the RNA is immobilised on a solid support and the solid support is contacted with the solution. In a further embodiment, the method comprises contacting a solid support with a biological sample containing the RNA; transferring the solid support or a portion thereof to a reaction vessel (wherein the portion comprises at least some of the RNA) optionally after a washing step; and performing the RT-PCR reaction in the reaction vessel in the solution in the presence of the solid support. The washing step, if used, comprises washing the solid support with an appropriate solution.

In a further embodiment, the solid support: (a) is fibrous, optionally comprising a cellulose fibre material or a glass fibre or glass microfiber material; or (b) comprises a porous polymer, optionally a porous membrane material such as polyester, polyether sulfone (PES), polyamide (Nylon), polypropylene, polytetrafluoroethylene (PTFE), polycarbonate, cellulose nitrate, cellulose acetate or alginate; or (c) comprises aluminium oxide.

In a further embodiment, a lysis reagent is embedded on the solid support. In a further
embodiment, the solid support is impregnated with one or more of (i) a weak base; (ii) a chelating agent; (iii) an anionic surfactant and (iv) a chaotropic agent such as guanidium thiocyanate.

According to a second aspect of the present invention, there is provided a dried reagent composition for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), the composition comprising a sequestering reagent; a polymerase; a deoxyribonucleotide triphosphate (dNTP); a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant.

In one embodiment, the dried reagent composition comprises a sequestering reagent; a polymerase; a deoxyribonucleotide triphosphate (dNTP); a serum albumin; a betaine; a non-ionic surfactant; and optionally a polar aprotic solvent.

In one embodiment, the sequestering agent is cyclodextrin. In a further embodiment, the serum albumin is bovine serum albumin (BSA) and the polar aprotic solvent, if present, is DMSO (dimethyl sulfoxide). In a further embodiment, the non-ionic surfactant is Triton X-100, Brij 56 or Brij 58, and/or the betaine is \( N,N,N \)-trimethylglycine. In a further embodiment, the dried reagent comprises the polar aprotic solvent.

According to a third aspect of the present invention, there is provided a method for producing a dried reagent composition of the present invention for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), comprising the steps: (a) combining a sequestering reagent; a polymerase; a dNTP; a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant to provide a mixture thereof; and (b) drying the mixture.

In one embodiment of this third aspect, the drying step is achieved by lyophilizing. In a further embodiment, the method comprises freezing said composition prior to the drying step.

According to a fourth aspect of the present invention, there is provided method of amplifying an RNA molecule comprising the steps: (i) incubating the RNA molecule and the dried reagent composition of the present invention in a solution; and (ii) performing a reverse transcription PCR (RT-PCR) reaction.

In one embodiment of this method, the solution comprises DMSO that is not derived from the dried reagent composition. In a further embodiment, the dried composition
does not comprise DMSO.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a picture of a representative agarose gel showing RT-PCR results indicating that inhibition of nucleic acid amplification was observed at blood concentration levels > 0.6% (v/v). Blood concentrations ranged from 1.25% to 0.03% (v/v) and total blood RNA was added to the test samples. The degree of inhibition was assessed by the generation of the appropriately sized β-globin RT-PCR product (291bp). 1 - genomic DNA; 2 - 1.25% (v/v) blood; 3 - 1.25% (v/v) blood supplemented with blood RNA (200ng); 4 - 0.6% (v/v) blood; 5 - 0.6% (v/v) blood supplemented with blood RNA (200ng); 6 - 0.3% (v/v) blood; 7 - 0.3% (v/v) blood supplemented with blood total RNA (200ng); 8 - 0.125% (v/v) blood; 9 - 0.125% (v/v) blood supplemented with blood RNA (200ng); 10 - 0.06% (v/v) blood; 11 - 0.06% (v/v) blood supplemented with blood RNA (200ng); 12 - 0.03% (v/v) blood; 13 - 0.03% (v/v) blood supplemented with blood RNA (200ng); 14 - Negative control and 15 - Positive control (total blood RNA, 200ng).

Figure 2 shows RT-PCR products generated using 0.03% (v/v) blood supplemented with blood RNA (200ng). These RT-PCR products are generated in the presence of differing SDS:cyclodextrin molar ratios. M - DNA markers; 1- human genomic DNA; 2 - Blood plus total RNA; 3 - Blood plus total RNA SDS:CD 1:1; 4 - Blood plus total RNA SDS:CD 1:1.5; 5 - Blood plus total RNA SDS:CD 1:2 and (+) - positive control total RNA only.

Figure 3 shows the results of direct RT-PCR using RT-PCR RTG beads (GE Healthcare), and various concentrations of anti-coagulated blood, random hexamers and β-globin primers. 1 - Blood (0.1% v/v) plus RNA (200ng); 2 - Blood (0.1% v/v); 3 - Blood (0.05% v/v) plus RNA (200ng); 4 - Blood (0.05% v/v); 5 - Blood (0.025% v/v) plus RNA; 6 - Blood (0.025% v/v); 7 - Blood (0.013% v/v) plus RNA; 8 - Blood (0.013% v/v) (+) - Positive control and (-) - Negative control. RT-PCR products derived from endogenous blood RNA are visible in lanes 6 and 7.

Figure 4 shows the results of direct RT-PCR using anti-coagulated 0.1% blood (v/v). The amplicons shown were generated using RT-RTG beads, random hexamers, β-globin primers in combination with increasing amounts of BSA. Lane 1 - 0.1% blood, no BSA plus RNA (200ng); 2 - 0.1% (v/v) blood, no BSA no RNA; 3 - 0.1% (v/v) blood, 0.3% (w/v) BSA plus RNA (200ng); 4 - 0.1% (v/v) blood, 0.3% (w/v) BSA no RNA; 5 - 0.1% (v/v) blood, 0.6% (w/v) BSA plus RNA (200ng); 6 - 0.1% (v/v) blood, 0.3% (w/v) BSA no RNA; 7 - 0.1% (v/v) blood, 0.9% (w/v) BSA plus RNA (200ng); 8 - 0.1% (v/v)
blood, 0.9 % (w/v) BSA no RNA; 10 - 0.1 % (v/v) blood, 1.2% (w/v) BSA plus RNA (200ng); 10 - 0.1 % (v/v) blood, 1.2% (w/v) BSA no RNA; M - DNA molecular weight markers, (+) and (-) positive and negative controls respectively. RT-PCR products are generated in the absence of added RNA (see lanes 4, 6, 8 & 10).

Figures 5 to 8 show graphs of actual versus predicted values to demonstrate R² (reflecting correlation), and results from Prediction Profiler. Figure 5 - Model 1; BSA 1% (w/v), DMSO 5% (v/v), Triton X-100 0.5% (v/v) and betaine (1 M). Least squared model; Average score of 3.77; R² = 0.93. Figure 6 - Model 2; BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10 mM). Least squared model; Average score of 3.29; R² = 0.91. Figure 7 - Model 3; BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v). Least squared model; Average score of 3.26; R² = 0.63. Figure 8 - Model 4; BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) and DTT 4.1 mM. Neural Net Model; Average score of 3.00; R² = 0.58.

Figure 9 shows the results of a performance validation of statistical models 1, 2 & 3. Representative agarose gels show the yield of RT-PCR products generated from the addition of the specific combinations of chemical to the RT-PCR RTG beads. These combinations were identified by PredictionProfiler using the SAS JMP software. Model 1 - BSA 1.0% (w/v), DMSO 5% (v/v), Triton 0.5% (v/v) & Betaine (1 M); Model 2 - BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10 mM), Model 3 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v), Control - BSA 0.6% (w/v), M = DNA molecular weight markers and (-) negative RT-PCR control.

Figure 10 shows the results of a performance validation of statistical model 4. Representative agarose gels show the yield of the RT-PCR product generated from the addition of the specific combinations of chemicals as defined in Model 4 to the RT-PCR RTG beads. This combination was identified by Prediction Profiler using the SAS JMP software. Model 4 - BSA 0.6% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (4.1 mM). Control - BSA 0.6% (w/v), M = DNA molecular weight markers and (-) negative RT-PCR control.

Figure 11 shows a representative gel showing the yield of β-actin RT-PCR products (626 bp) derived from HeLa cells applied to FTA sample collection cards using RT-PCR RTG beads supplemented with specific combinations of chemicals. These combinations were identified by the PredictionProfiler using the SAS JMP software during the DOE evaluating. Model 1 - BSA 1.0% (w/v), DMSO 5% (v/v), Triton 0.5% (v/v) & Betaine (1 M), Model 2 - BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10
mM), Model 3 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v) Model 4 - BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) and DTT 4.1 mM, Control - BSA 0.6% (w/v), M = DNA molecular weight markers and (−) negative RT-PCR control. Increased RT-PCR product yield was observed from all reactions supplemented with the chemical additives as described in Tables 3 & 4.

Figure 12 shows a representative composite agarose gel showing the yield of β-globin RT-PCR products generated using diluted blood in combination with the addition of the specific chemicals to "liquid" RT-PCR reactions. These chemical combinations were identified by the Prediction profiler using the SAS JMP software and during the DOE evaluating the use of RT-PCR RTG beads. Model 1 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v); Model 2 - BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10 mM); Model 3 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v), Control - BSA 0.6% (w/v), M = DNA molecular weight markers and (−) negative RT-PCR control.

Figure 13 shows a representative composite agarose gel showing the yield of RT-PCR products generated using HeLa cell applied to FTA Sample collection cards in combination with the addition of the specific chemicals to "liquid" RT-PCR reactions. These chemical combinations were identified by the Prediction profiler using the SAS JMP software and during the DOE evaluating the use of RT-PCR RTG beads. Model 1 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v) and Model 3 - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v); are shown as an illustration, Control - BSA (0.6% w/v), M = DNA molecular weight markers and (−) negative RT-PCR control.

**DETAILED DESCRIPTION OF THE INVENTION**

The present application is directed to methods and reagents for use amplifying RNA by reverse-transcription-PCR (RT-PCR). Traditional RT-PCR based assays rely on RNA extraction prior to amplification, thus removing any potential RT-PCR inhibitors present in the biological material. As highlighted above, this extraction or purification step is associated with several disadvantages. Accordingly, the present inventors carried out extensive studies to identify particular combinations of reagents and/or method steps that would allow effective RT-PCR reactions to be carried out on crude RNA samples, without the need to carry out prior-steps aimed at extracting and/or purifying RNA from the sample. The present inventors set about this goal by researching many different combinations of reagents for use in the RT-PCR reaction. The present inventors further identified optimisation steps up-stream of the RT-PCR reaction that can be employed to
further enhance the process.

Accordingly, in a first aspect, the present invention relates to a method of amplifying an RNA molecule in a biological sample by reverse transcription PCR (RT-PCR), wherein the RT-PCR is carried out in a solution comprising a polar aprotic solvent; a serum albumin; and optionally a non-ionic surfactant and/or a betaine.

RT-PCR is an RNA amplification technique that is well known in the art. In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for amplification using PCR. The use of RT-PCR for the detection of RNA transcript has revolutionized the study of gene expression. In RT-PCR, the cDNA is generated through the action of a reverse transcriptase. Enzymes for use in this step are well known in the art. The cDNA sequence may be generated from the full length of the mRNA sequence or a portion of the mRNA sequence. Accordingly, first-strand cDNA synthesis reactions can use oligo-dTs (short sequences of deoxy-thymine nucleotides as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand). Alternatively, random primers or sequence specific primers may be employed in this step. A skilled person can readily determine the appropriate annealing and extension temperatures from the primer sequence, mRNA template and choice of reverse transcriptase using procedures well known in the art.

The amplification step comprises performing a polymerase chain reaction (PCR) on the generated cDNA sequence. It should be noted that references throughout this disclosure to amplifying a cDNA sequence encompasses amplification of either the complete cDNA sequence generated in the reverse-transcription step or a part of the cDNA sequence generated in the reverse transcription step. (PCR) is widely known in the art. For example, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; K. Mullis, Cold Spring Harbor Symp. Quant. Biol., 51:263-273 (1986); and C. R. Newton & A. Graham, Introduction to Biotechniques: PCR, 2.sup.nd Ed., Springer-Verlag (New York: 1997), the disclosures of which are incorporated herein by reference, describe processes to amplify a nucleic acid sample target using PCR amplification extension primers which hybridize with the sample target. Using PCR, the cDNA is amplified exponentially using a polymerase e.g. a DNA polymerase. PCR requires forward and reverse extension primers which hybridize with the sample target. As the PCR amplification primers are extended, using a DNA polymerase (preferably thermostable), more sample target is made so that more primers can be used to repeat the process, thus amplifying the sample
target sequence. Typically, the reaction conditions are cycled between those conducive to hybridization and nucleic acid polymerization, and those that result in the denaturation of duplex molecules. To briefly summarize, in the first step of the reaction, the nucleic acid molecules of a sample are transiently heated, in order to denature double stranded molecules. Forward and reverse primers are typically present in the amplification reaction mixture at an excess concentration relative to the sample target. When the sample is cooled to a temperature conducive to hybridization and polymerization, the primers hybridize to the complementary sequence of the nucleic acid molecule at a position 3’ to the sequence of the region desired to be amplified that is the complement of the sequence whose amplification is desired. Upon hybridization, the 3’ ends of the primers are extended by the polymerase. The extension of the primer results in the synthesis of a DNA molecule having the exact sequence of the complement of the desired nucleic acid sample target. The PCR reaction is capable of exponentially amplifying the desired nucleic acid sequences, with a near doubling of the number of molecules having the desired sequence in each cycle. Thus, by permitting cycles of denaturation, hybridization, and polymerization, an exponential increase in the concentration of the desired nucleic acid molecule can be achieved. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denaturated. Typical heat denaturation involves temperatures ranging from about 80°C to about 105°C, for times ranging from a few seconds to minutes.

In the present invention, the template for amplification is the cDNA strand produced during the reverse transcription step. Accordingly, the PCR reaction would typically require a forward primer that anneals to the cDNA strand produced during the reverse transcription step and which is then extended using an enzyme with DNA polymerase activity to produce the complement cDNA strand. The resulting cDNA strand can then be denatured and the forward primer and a reverse primer annealed to the respective cDNA strands to allow further extension. The primers are then extended by the polymerase to replicate the cDNA sequences, and the process is then repeated multiple times.

The cocktail of reagents used for the RT-PCR reaction may comprise a reverse transcriptase enzyme for the reverse transcription step and a DNA polymerase enzyme for the PCR reaction. However, a single enzyme may also be used that is able to perform the enzymatic steps in both the reverse transcription reaction and the PCR reaction. An example of such an enzyme that is configured for DNA polymerization and reverse transcription that can be used in the present invention is rTth. Other reagents include one or more primers (described above), dNTPs (deoxynucleotide triphosphates).
Suitable buffers may also be employed.

The term "primer" refers to a molecule that physically hybridizes with a target nucleic acid. The primer is capable of being extended in an amplification reaction such as a PCR reaction or in a reverse-transcription reaction. Typically, a primer can be made from, or comprise of, any combination of nucleotides or nucleotide derivatives or analogs available in the art. More typically, a primer will be in the form of an oligonucleotide. Primers may also contain one or more nucleotide alternatives or modified bases to add increased specificity and/or disrupt the efficiency of primer extension in the presence of a mis-match. Alternative bases used to enhance specificity may include Locked Nucleic Acid (LNA) bases, Peptide Nucleic Acid (PNA) bases and Inosine. The primer may be unlabelled or labelled with a detection marker.

The term "polar aprotic solvent" is used to denote a solvent that will dissolve many salts, has a comparatively high relative permittivity (or dielectric constant), e.g., greater than ca. 15, and a sizable permanent dipole moment, that cannot donate suitably labile hydrogen atoms to form strong hydrogen bonds. In a preferred embodiment, the polar aprotic solvent dissolves both polar and non-polar compounds. In a particularly preferred embodiment, the polar aprotic solvent used in the present invention is dimethyl sulfoxide (DMSO).

The polar aprotic solvent may be employed in the solution at a concentration in the range 0.05-10% (v/v). For example, the polar aprotic solvent may be used at a concentration in the range 1-9% (v/v), 2-8% (v/v), 3-7% (v/v) or 4.5-5.5% (v/v). Typically, the polar aprotic solvent may be used at a concentration of approximately 5% (v/v).

Serum albumin, often referred to simply as blood albumin, is a globular protein that in humans is encoded by the ALB gene. Serum albumin is produced by the liver, occurs dissolved in blood plasma and is the most abundant blood protein in mammals. The serum albumin that may be used in the present invention may be, for example, bovine serum albumin, human serum albumin, goat serum albumin, mammalian albumin, or any combination thereof. In a particularly preferred embodiment, the serum albumin is bovine serum albumin (BSA).

The serum albumin may be employed in the solution at a concentration in the range 0.05-1.2% (w/v). For example, the serum albumin may be used at a concentration in the range 0.1 - 1.2% (w/v), 0.2 - 1.2% (w/v), 0.3 - 1.2% (w/v), 0.4 - 1.2% (w/v), 0.5-1.2%
(w/v) or 0.6-1.2% (w/v). In a preferred embodiment, the serum albumin is used at a concentration in the range of 0.5 -1.1% (w/v).

In one embodiment the solution comprises a polar aprotic solvent and a serum albumin.

In one embodiment, the solution comprises a polar aprotic solvent, a serum albumin and a betaine. In one embodiment, the solution comprises a polar aprotic solvent, a serum albumin, a betaine and a non-ionic surfactant. In one embodiment, the solution comprises a polar aprotic solvent and a serum albumin but does not further comprise one or more of a betaine (e.g. N,N,N-trimethylglycine), a non-ionic surfactant (e.g. Triton X-100, Brij 56 and/or Brij 58) and a reducing agent (e.g. dithiothreitol (DTT) and/or Tris(2-carboxyethyl) phosphine (TCEP), including salts thereof).

The non-ionic surfactant may be employed to prevent the polymerase(s) sticking to themselves or the walls of the reaction tube (preventing loss of reagents through adsorption to tube walls). It may also aid in solubilizing the reaction containing the BSA, destabilizing secondary structures and stabilizing the polymerase (e.g. Taq polymerase). Non-ionic surfactants or detergents also have the added benefit of overcoming inhibitory effects of trace amounts of strong ionic detergents.

The non-ionic surfactant may be a non-ionic surfactant comprising a hydrophilic polyethylene oxide chain. The non-ionic surfactant may comprise both a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group such as 4-(l,l,3,3-tetramethylbutyl)-phenyl group. In a preferred embodiment, the non-ionic surfactant is 4-(l,l,3,3-Tetramethylbutyl)phenyl-polyethylene glycol.

The non-ionic surfactant may also be a polyethylene glycol hexadecyl ether of formula C16H33(OCH2CH2)nOH where n is between 5 and 25, preferably between 9 and 20, and preferably still 10 or 20.

In a particularly preferred embodiment, the non-ionic surfactant is Triton X-100, Brij 56 or Brij 58, or a combination thereof. In a most preferred embodiment, the non-ionic surfactant is Triton X-100.

The non-ionic surfactant may be employed in the solution at a concentration in the range 0.05-1% (v/v). For example, the non-ionic surfactant may be used at a concentration in the range 0.1 - 1% (v/v), 0.2-1% (v/v), 0.3-1% (v/v), 0.4-1% (v/v), 0.1-0.9% (v/v), 0.1-0.8% (v/v), 0.1-0.7% (v/v), 0.1-0.6% (v/v), 0.3-0.7% (v/v), or 0.4-0.6% (v/v). In a preferred embodiment, the non-ionic surfactant is used at a concentration of
approximately 0.5 % (v/v).

Betaines are a neutral chemical compounds with a positively charged cationic functional group such as a quaternary ammonium or phosphonium cation and which typically bear no hydrogen atom directly attached to the charged atom of the cation and with a further negatively charged functional group such as a carboxylate group. In the present invention, the betaine preferably reduces the formation of secondary structure in GC-rich regions by reducing the amount of energy required to separate nucleic acid strands, and/or reducing the base pair composition dependence of DNA melting. In a particularly preferred embodiment of the present invention, the betaine is N,N,N-trimethylglycine (also known as 2-trimethylammonioacetate).

The betaine may be employed in the solution at a concentration in the range 0.05-2M. For example, the betaine may be used at a concentration in the range 0.1-2M, 0.2-2M, 0.3-2M, 0.4-2M, 0.5-2M, 0.6-2M, 0.7-2M, 0.8-2M, 0.9-2M, 0.5-1.5M, 0.6-1.4M, 0.7-1.3M, or 0.8-1.2M. In a preferred embodiment, the betaine is used at a concentration of approximately 1 M.

Examples of preferred solutions for use in the present invention comprise: (a) DMSO at a concentration in the range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to 1.2% (w/v); Triton X-100 at a concentration in the range 0.05 to 1% (v/v); and N,N,N-trimethylglycine at a concentration in the range 0.05 to 2M; or (b) DMSO at a concentration in the range 0.05 to 10% (v/v), BSA at a concentration in the range 0.05 to 1.2% (w/v); or (c) DMSO at a concentration in the range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to 1.2% (w/v); and N,N,N-trimethylglycine at a concentration in the range 0.05 to 2M.

It will be appreciated that the solution will comprise other reagents necessary for performing a RT-PCR reaction, as discussed above. For example, the solution may further comprise a reverse transcriptase, a DNA polymerase, deoxyribonucleotide triphosphates (dNTPs); and at least one primer. As discussed above, the reverse transcriptase and the DNA polymerase may be the same enzyme (where the enzyme has the ability to carry out both reverse transcription and DNA amplification) or separate enzymes. A skilled person would recognise that further buffer reagents may also be employed, as is well known in the art.

The method of RT-PCR disclosed may be carried out on crude biological samples and/or biological samples that have not undergone RNA extraction or purification prior to the
RT-PCR reaction. In one embodiment, the biological sample has not undergone treatment with a DNase prior and/or has not undergone an RNA purification step prior to the RT-PCR reaction. An example of such a purification or extraction method is acid guanidinium thiocyanate-pheno-chloroform extraction. Other examples include column-based systems such as silica-based purification.

Accordingly, the method of RNA amplification of the present invention may be carried out with significantly less "pre-processing" of the sample compared to known methods. Furthermore, the RT-PCR can take place in a "one-step" approach, where the cDNA synthesis and the PCR amplification occurs in a single tube, and both the cDNA synthesis and the PCR amplification take place in the presence of a polar aprotic solvent, a serum albumin, a polyol, and optionally, as described above, one or more of a reducing agent, a non-ionic surfactant and a betaine. However, it will be appreciated that whilst the RT-PCR reaction takes place in a solution comprising these components, further substances/solutions may be added to the solution during the reaction (e.g. between initiating the reverse transcription reaction and the performing the PCR reaction) and the invention scope will be understood to cover such additional steps. In one embodiment, no further substances/solutions are added to the solution during the reaction.

Prior to all or part of the RT-PCR reaction, the biological sample may be subjected to a lysis step. Preferably, prior to all or part of the RT-PCR reaction, the biological sample is subjected to a lysis step and a detergent neutralization. The detergent neutralisation step neutralises the detergent used in the lysis step. The lysis step is performed by contacting the biological sample with a detergent, preferably sodium dodecyl sulphate (SDS), and the detergent neutralization step is preferably performed by contacting the sample with a detergent sequesterant, preferably a cyclodextran. The cyclodextran may, for example, be selected from the group consisting of one or more of α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, 6-O-a-D-Maltosyl-β-cyclodextrin, hydroxyethyl-β-cyclodextrin, hydroxypropyl-P-cyclodextrin and 2-hydroxypropyl-P-cyclodextrin or derivatives thereof. In one embodiment, the cyclodextrin is selected from the group consisting of α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin. In one embodiment, the cyclodextrin is a-cyclodextrin.

The biological sample may comprise a cellular sample selected from the group consisting of blood, serum, semen, cerebral spinal fluid, synovial fluid, lymphatic fluid, saliva, buccal, cervical cell, vaginal cell, urine, faeces, hair, skin, muscle, and cells grown in culture. The biological sample may be derived from a virus, a eukaryotic organism or a prokaryotic organism.
In a preferred embodiment, the biological sample is a blood sample. The blood sample may be a blood sample that has been treated with an anti-coagulant. Anticoagulants suitable for use in the present invention include heparin and substances that make Ca\textsuperscript{2+} unavailable for clotting (e.g., EDTA, citrate, oxalate, fluoride). Preferred examples include sodium heparin, a potassium oxalate and sodium fluoride combination, EDTA and sodium citrate.

The RNA in the biological sample may be immobilised on a solid support such that the solid support, or a portion of the solid support comprising at least some of the RNA, is contacted with the solution in order to perform the RT-PCR reaction.

Solid supports for storing, transporting and archiving of nucleic acids such as filter paper or chemically modified matrices are well-known in the art. Furthermore, solid supports are commercially available that can be used directly in nucleic acid amplification reactions. Examples of solid supports are described in, for example, US 2014/0212880 Al, US 2014/0154667 Al, EP1563091 Al, WO1990003959, US5496562, the entire contents of which are incorporated herein.

The solid support may be fibrous, optionally comprising a cellulose fibre material or a glass fibre or glass microfiber material. The solid support may comprise a porous polymer, optionally a porous membrane material such as polyester, polyether sulfone (PES), polyamide (Nylon), polypropylene, polytetrafluoroethylene (PTFE), polycarbonate, cellulose nitrate, cellulose acetate or alginate. In a preferred embodiment, the solid support is a cellulose-based matrix. The solid support may comprise a lysis reagent and a sequestering reagent. The solid support may be impregnated with a weak base; a chelating agent; an anionic surfactant and a chaotropic agent such as guanidium thiocyanate. Examples of suitable commercially available matrices are in the form of FTA\textsuperscript{TM} and FTA\textsuperscript{TM} Elute card (GE Healthcare). The matrix may be in the form of a pre-punched disc. Where a solid support is employed, the RT-PCR reaction may take place at the surface of the solid support whilst the solid support is present in the solution.

The method of the invention can be used, for example, either in a single reaction well or a high-throughput 96-well format in combination with automated sample processing as described by Baron et al., (2011, Forensics Science International: Genetics Supplement Series, 93, e560-e561). This approach would involve a minimal number of steps and increase sample throughput. The risk of operator-induced error, such as cross-contamination is also reduced since this procedure requires fewer manipulations.
compared to protocols associated with currently used, more labour intensive kits (e.g. QIAmp DNA blood mini kit, Qiagen). The risk of sample mix-up is also reduced since the procedure requires few manipulations. Importantly, the method is readily transferable to a multi-well format for high-throughput screening. The present invention can thus improve sample processing for carrying out PCR reactions to aid genetic interrogations. The invention can be conducted in a 96 well/high throughput format to facilitate sample handling and thus eliminate batch processing of samples.

The findings of the present invention can be utilized to generate improved dried reagent compositions for use in amplifying an RNA molecule by RT-PCR. Dried reagent compositions for use in RT-PCR are commercially available, for example, the illustra Ready-To-Go RT-PCR beads (GE Healthcare). These Ready-To-Go RT-PCR Beads are stable at room temperature and designed for performing single-tube one-step reverse transcription-PCR. Each room-temperature-stable bead contains M-MuLV Reverse Transcriptase, RNase Inhibitor, buffer, nucleotides, and Taq DNA Polymerase. The only additional reagents that need be added to the beads to perform the RT-PCR are water, template RNA, and primers.

The Ready-To-Go Bead format significantly reduces the number of pipetting steps, thereby increasing reproducibility of the RT-PCR technique and minimizing risk of contamination and RNA degradation. Ready-To-Go RT-PCR Beads are provided in either thin walled 0.5 ml or 0.2 ml tubes compatible with most thermocyclers. The 0.2 ml tubes come assembled in a 96-well (8 x 12) plate format that allows individual strips of eight tubes to be easily removed. This flexibility allows use of either the entire 96-well plate, strips of eight or individual 0.2 ml tubes. Each package of Ready-To-Go RT-PCR Beads contains: RT-PCR beads, control reactions and pd(N)6 and oligo(dT) cDNA primers.

Further examples of the use of dried compositions (e.g. in the form of beads or cakes) for use in PCR reactions, and methods of making such compositions, are described in WO2014064169 A1, US5593824, EP2063866 B1 and US5565318, the entire contents of which are incorporated herein.

Accordingly, in a second aspect, the invention provides a dried reagent composition for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), the composition comprising a sequestering reagent; a polymerase; a deoxyribonucleotide triphosphate (dNTP); a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant.
The serum albumin, betaine, non-ionic surfactant and polar aprotic solvent may be chosen from any of the embodiments described above with respect to the first aspect of the invention. In a preferred embodiment the polar aprotic solvent, if present, is DMSO, the serum albumin is BSA; the non-ionic surfactant, if present, is Triton X-100, Brij 56 or Brij 58, and the betaine is N,N,N-trimethylglycine.

The dried reagent composition may comprise, for example: a) DMSO; and BSA; b) DMSO; BSA; and N,N,N-trimethylglycine; or c) DMSO; BSA; Triton X-100, Brij 56 or Brij 58; and N,N,N-trimethylglycine.

The enzyme performing the reverse transcriptase activity and the polymerase activity may be the same or different enzymes. As mentioned above, a single enzyme may also be used that is able to perform the enzymatic steps in both the reverse transcription reaction and the PCR reaction. In a preferred embodiment, the reverse transcriptase and the DNA polymerase are different enzymes.

In one embodiment, the polymerase is an OmniKlen Taq (OKT) Polymerase. Alternatively, the polymerase may be selected from the group consisting of T4 DNA Polymerase, Pol I and Klenow Fragment, T4 DNA Polymerase, Modified Bacteriophage T7 DNA Polymerase, Terminal Deoxynucleotid Transferase, Bst Polymerase, Taq Polymerase, Th polymerase, Pov Polymerase, Vent Polymerase, Pab Pol I DNA Polymerase, Thermus thermophiles, Carboxydothentrus hydrogenoformans, SP6 and SP7 RNA polymerase. In a further embodiment, the reverse transcriptase is M-MuLV Reverse Transcriptase, and the polymerase is Taq DNA Polymerase.

In one embodiment, the sequestering agent is a cyclodextrin. The cyclodextrin may be selected from a group consisting of α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin and derivatives thereof. Cyclodextrin could consist of a group consisting of 6-O-a-D-Maltosyl-β cyclodextrin, hydroxyethyl -P-cyclodextrin, hydroxypropyl -P-cyclodextrin and 2-hydroxypropyl -P-cyclodextrin. The sequestrant is preferably a-cyclodextrin. The sequestering reagent is preferably not a chelating agent. A chelating agent is a chemical compound that combines with a metal to form a chelate, often used to trap heavy metal ions (Collins English Dictionary, © HarperCollins Publishers 2003).

In a further embodiment, the dried reagent composition comprises a least one primer.

In a further embodiment, the dried reagent composition additionally comprises an
excipient mix. The term "excipient mix" is used herein to denote additives or ingredients used to make up a preparation or mixture and for example may comprise of PCR buffer, Ficoll 70, Ficoll 400, Melezitose, Trehalose, and stabilising proteins.

The term "PCR buffer" is used herein to denote a buffer necessary to create optimal conditions for activity of a DNA polymerase and/or a reverse transcriptase, and for example may comprise of Tris-HCl, KCl, MgCl₂, and gelatin.

In a further embodiment, the dried reagent composition additionally comprises an exchange buffer or buffer composition. The term "exchange buffer" is used herein to denote a buffer used for the removal of small ionic solutes, whereby one buffer is removed and replaced with another alternative buffer and for example may comprise of Tris/HCl, CaCl₂, a detergent, RE960, MgCl₂, and KCl.

The RT-PCR dried reagent composition may typically contain buffer, dATP, dCTP, dGTP, dTTP, Taq DNA Polymerase, M-MuLV reverse transcriptase, and an RNase inhibitor. The composition may further comprise one or more stabilizers. The dried composition, e.g. a bead or cake, may be formulated such that, when it is reconstituted (e.g. to a volume of 50 μl), the concentration of each dNTP is approximately 200 μM in approximately 10 mM Tris-HCl (approximately pH 9.0), approximately 60 mM KCl, and approximately 1.5 mM MgCl₂.

The concentration of polar aprotic solvent, serum albumin, non-ionic surfactant and betaine (to the extent they are present in the dried composition) may be such that, when the dried composition is incubated in a volume of solution for the RT-PCR reaction (e.g. in the range 10 to 200 μl, such as 20 to 100 μl, and preferably approximately 50 μl), these components achieve the concentration levels set out above with respect to the first aspect of the invention.

In a further embodiment, the dried reagent composition is a lyophilized composition. The advantage of dried or lyophilised formulations of the RT-PCR reagents is that they can be easily solubilised by the addition of water, thus saving operator time and facilitating operator usage. To minimise operator error, the dried reagent mixture can be pre-dispersed into the reaction vessel, such as the well of a multi-well plate. The preformulated, predispensed, ambient-temperature-stable beads or cakes allow amplification reactions to be carried out within a single well or reaction vessel and ensure greater reproducibility between reactions, minimize pipetting steps, and reduce the potential for pipetting errors and contamination.
The dried reagent composition can be used, for example, either in a single reaction well or a high-throughput 96-well format in combination with automated sample processing as described above.

In one embodiment, the dried reagent composition comprises DMSO. In another embodiment, the dried reagent composition does not comprise DMSO and the DMSO is added as part of the RT-PCR reaction mixture that is added to the dried composition for carrying out the RT-PCR reaction.

In a third aspect, the present invention provides a method for producing a dried reagent composition as defined in the second aspect for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), comprising the steps: combining a polymerase; a sequestering reagent; a dNTP; a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant, to provide a mixture thereof; and drying the mixture.

The drying step is preferably achieved by lyophilizing the mixture. The mixture may be subjected to a freezing step prior to the drying step.

It will be appreciated that other ingredients may be included in the mixture of the third aspect, for example one or more of the further ingredients that may be present in the dried composition of the second aspect of the invention, and the inclusion of such further ingredients in the mixture described in the third aspect of the invention shall be considered to be within the scope of the method of the third aspect.

In a fourth aspect, the present invention provides a method of amplifying an RNA molecule comprising the steps: (i) incubating the RNA molecule and the dried reagent composition of the second aspect of the present invention; and (ii) performing a reverse transcription PCR (RT-PCR) reaction.

In step (i) of the fourth aspect, water is added to the RNA molecule. Step (i) may also comprise adding primers for the reverse transcription reaction and/or PCR reaction. Step (i) may also comprise adding the non-polar aprotic solvent, such as DMSO, for example where the dried reagent does not comprise DMSO. The RNA molecule may be part of a biological sample described above. The RNA molecule may be immobilised on a solid support as described above. The RNA molecule may be part of a biological sample that has not undergone treatment with a DNase prior to the RT-PCR reaction and/or has not
undergone an RNA purification step prior to the RT-PCR reaction.

The present invention will now be described with reference to the following non-limiting examples.

**Experimental outline**

The aim of this study was to identify a reverse transcriptase-polymerase chain reaction (RT-PCR) formulation which would directly amplify a RT-PCR product from the RNA present in crude biological samples. Experimentation focussed on the amplification of RNA directly from diluted whole blood, cultured cells and biological samples applied to chemically-coated FTA sample collection cards (GE Healthcare) using liquid and freeze-dried reagents such as the illustra Ready-to-Go (RTG) RT-PCR reagent (GE Healthcare). Whole blood is considered to be a difficult source from which to amplify nucleic acids due to the presence of multiple inhibitors. The illustra RTG beads provide temperature stable reagents designed for performing single-tube one-step RT-PCR. Each room temperature-stable bead contains M-MuLV Reverse Transcriptase, RNase inhibitor, buffer, nucleotides, and wildtype Taq DNA Polymerase. The only additional reagents required are water, RNA, and the appropriate primers and these are supplied by the user depending upon their specific application.

Initially, experimental work focussed on the development of an RT-PCR protocol in liquid formulations using Affinity-Script RT, and the mutated taq DNA polymerase Omniklentaq in combination with the PCR enhancer cocktail PEC-1. Omniklentaq is an engineered enzyme that is considered to be inhibitor resistant. This initial work attempted the amplification of RNA directly from whole EDTA-treated blood samples and investigated a range of blood concentrations. Results (not shown) demonstrated that Omniklentaq in combination with PEC-1 alone was not able to overcome RT-PCR inhibition when using blood as the RNA source even at extremely low blood concentrations [final blood diluted as low as 0.03% v/v with phosphate buffered saline (PBS)].

Later experiments focussed on developing i) an efficient detergent-based cellular lysis method suitable for direct RT-PCR and ii) a model system that enabled the amplification of RNA from diluted blood from which a more efficient system could be developed. Results from initial experimentation showed that RNA could be successfully amplified from up to 0.1% v/v blood using a combination of RTG RT-PCR beads with the addition of BSA 0.6 (w/v). Therefore the combination of diluted blood and the RT-PCR RTG
beads demonstrated that RT-PCR products could be generated albeit at a relatively low yield.

In order to increase direct RT-PCR product yield, the effect on nucleic acid amplification of a number of chemicals was investigated. A formal statistically-relevant Design of Experiment (DOE) was performed and the results highlighted several combinations of chemicals that generated significantly higher yields of RT-PCR product compared a control system. Therefore, RT-PCR product yield could be increased by adding specific combinations of chemical to the RT-PCR RTG bead formulation. Later these combinations were also shown to deliver increased RT-PCR product yield when used in different formats including a liquid RT-PCR formulation and the direct amplification of RNA molecules from blood, tissue and biological material applied to solid supports such as GE Healthcare/Whatman FTA sample collection cards.

### Materials

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### Direct RT-PCR method

A RT-PCR process was initially performed using AffinityScript RT (Agilent Technologies), Omniklentaq DNA polymerase and PCR enhancer cocktail 1 (DNA Polymerase Technology). The reaction mixture consisted of EDTA anti-coagulated human blood diluted to 1.25% to 0.03% (v/v), with PBS either in the presence (200 ng) or absence of total RNA, and either Oligo (d)T or random hexamers. Purified total RNA was derived from either human blood (Clontech) or total RNA extracted from human HeLa cells using the illustra RNAspin kit (GE Healthcare) following the manufacturer's instructions. Before use the RNA was incubated at 65 °C for 5 min then allowed to cool at room temperature for 10 minutes.

Following the incubation, an AffinityScript RT reaction was established according to the manufacturer's recommendation. This consisted of xiO buffer, DDT, 100 mM dNTPS and AffinityScript RT enzyme (2 units). The RT reaction was performed at 42 °C for 60 min followed by an enzyme heat inactivation step of 70 °C for 15 min.

For the PCR reaction, thermocycling conditions were; 94°C, 4 min, followed by 32 cycles of 94°C, 1 min, 55°C, 1 min and 68°C 2.5 min. To differentiate amplified PCR products derived from RNA and genomic DNA, primers were designed at exon boundaries to amplify a 626 bp, 291 bp or 258 bp RT-PCR products from the RNA encoding human β-actin, β-globin or GAPDH genes respectively. PCR Primer sequences used were: β-globin, exon I forward 5′-GGT GAA CGT GGA TGA AGT TG-3′ and exon III reverse 5′-AGC ACA CAG ACC AGC ACG T-3′; β-actin, exon 1 forward 5′-CCTCGCCTTTGCCGATCC-3′ and exon 4 reverse 5′-GGATCTTCATGAGGTAGTCAGTC-3′; GAPDH forward 5′-
AGAAGGCTGGGGCTC ATTTG-3' and reverse 5' AGGGGCCATCCACAGTCTTC-3'.

OmniKlenTaq DNA polymerase and the recommended PEC 1 was used according to manufacturer's instructions. The mutated OmniKlenTaq DNA polymerase when used with PEC 1 has the ability to tolerate many inhibitors commonly found in biological samples. However, during these experiments it was shown that the combination of AffinityScript RT, OmniKlenTaq and PEC 1 failed to generate RT-PCR products irrespective of the blood concentration used.

During a separate evaluation and according to manufacturer's claims it was confirmed that OKT polymerase and PEC 1 were able to amplify PCR products directly from genomic DNA contained in whole blood in both liquid and freeze dried RTG formats (data not shown).

**Optimising the concentration of blood**

To determine the most appropriate blood concentration that could support a direct RT-PCR reaction a range of blood dilutions, 1.25% to 0.03% (v/v), were generated using PBS. The aim was to identify the most appropriate concentration of blood that did not exhibit any inhibition on direct RT-PCR amplification. As a control, RNA (200ng) isolated from whole blood or purified from HeLa cells were added to the diluted blood and RT-PCR was performed using β-globin, GAPDH and β-actin primers respectively.

Blood total RNA was purchased from Clontech and HeLa cells RNA was extracted using the illustra RNAspin kit (GE Healthcare). The quality of the RNA was assessed using 2% agarose gel electrophoresis confirming the presence of the 28S, 18S and the faint 5S ribosomal RNA. RNA concentration was determined using the Nanovue spectrophotometer (GE Healthcare). Results confirmed that the RNA derived from both whole blood and HeLa cells was of sufficient quality for RT-PCR. The OmniKlenTaq and PEC-1 system used initially was replaced with a cloned version of wildtype Taq DNA polymerase (GE Healthcare) in combination with AffinityScript RT following the protocol described above.

Using this alternative RT-PCR system, direct RT-PCR products were generated (see Figure 1). However amplicons were observed from only samples containing blood concentrations < 0.06% (v/v) and to which RNA was added (Figure 1, Lanes 11 and 13). Therefore, these data indicate that even relatively low blood concentrations contain RT-
PCR inhibitors. The optimal conditions for reducing the effect of potential RT-PCR inhibitors was identified as < 0.03% (v/v) blood (lane 13) when using the wildtype Taq DNA polymerase.

Similar results were observed when HeLa total RNA was added to diluted blood in combination with β-actin and GAPDH primer sets (data not shown). Based upon these results wildtype Taq DNA polymerase and AffinityScript RT was used in all subsequent liquid based RT-PCR reactions.

**Optimised Cellular Lysis using SDS and CD**

The use of SDS to lyse cells followed by a treatment with cyclodextrin (CD) as a detergent sequesterant is known (Horton, J.K., 1997, In-situ cell extraction and assay method, EP 0863402 B1). Experiments were conducted to investigate if SDS and cyclodextrin could be used to disrupt cells and potentially improve the liberation and survival of RNA by denaturing and inactivating proteins and enzymes including ribonucleases (RNases), whilst at the same time not exhibiting any inhibitory effect on the direct RT-PCR amplification reaction.

The performance of different SDS:CD ratios were compared using blood diluted with PBS to a concentration range of 1.25% to 0.03% (v/v). To represent, and as a means of illustration, the results derived from the use of 0.03% (v/v) blood is shown (Figure 2). Cells from EDTA anti-coagulated blood were lysed by the addition of SDS to a final concentration of a 2% (w/v) and incubated at room temperature for 5 min. The SDS was neutralised by the addition of freshly prepared CD at molar ratios of SDS:CD 1:1, 1:1.5 and 1:2.

RT-PCR was performed using the β-globin specific primers. All blood samples were spiked with RNA (200ng) derived from white blood cells. A positive control was performed using purified blood total RNA. This study was designed to optimise the ratio of SDS:CD and to determine if using the SDS and CD system had any inhibitory effect on the RT-PCR reaction.

The correct β-globin PCR product (291 bp) was generated from only those blood samples which contained the added RNA and had been subjected to the SDS and CD treatment (see Figure 2). PCR products were not generated either in the absence of SDS which is a potent protein denaturant or when using only genomic DNA. In the absence of added RNA, samples failed to generate the RT-PCR products. Based upon these
results all subsequent experiments used a SDS:CD molar ratio of 1:2. These surprising results demonstrate that the SDS:CD system can be used to significantly improve the efficacy of RT-PCR reactions.

5 RT-PCR using RT-PCR RTG beads

Due to the successful amplification of RT-PCR products using wildtype Taq DNA polymerase, the use of GE Healthcare RT-PCR RTG beads was evaluated. These beads contain the same Taq DNA polymerase used in the previous experiments but contain M-MuLV Reverse Transcriptase. These enzymes are stabilised using an excipient mixture in a freeze-dried format supplemented with all the reagents required for RT-PCR.

RT-PCR reactions were performed using SDS:cyclodextrin blood lysis system described earlier and the RT-PCR RTG beads according to manufacturer’s recommendations. RT-PCR RTG beads were placed on ice and resuspended in RNase free water. EDTA anticoagulated blood was diluted with PBS to a concentration range of 1.25% to 0.03% (v/v), total RNA (200ng) derived from blood or HeLa cells and either oligo d(T) or random hexamers were added. Prior to use the RNA was incubated at 65 °C for 5 min then allowed to cool at room temperature for 10 minutes. To initiate the RT reaction all reagents were mixed and incubated at 42 °C for 60 minutes, followed by 95 °C for 3 minutes. PCR-primer sets for β-actin, β-globin and GAPDH was used and the following thermocycle was performed for 32 cycles at 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 1 minutes with an extension of 72 °C for 5 minutes.

Direct RT-PCR products were successfully generated when using RT-PCR RTG beads with random primers and oligo dT. This was in combination with diluted blood at a concentration of 0.1% (v/v) and supplemented with RNA (Figure 3). A visual inspection of the agarose gel appears to indicate that the amount of product generated increases with decreasing blood concentrations. This implies that even at low blood dilutions [0.1% (v/v)] a degree of RT-PCR inhibition is still present, however, the use of RT-PCR RTG beads appears to overcome a portion of this inhibitory effect. Encouragingly, direct RT-PCR products were visible (albeit at low amounts) when using < 0.025% (v/v) blood in the absence of added RNA (see Figure 3, lanes 6 & 7). This indicates that the RT-PCR products are being amplified directly from the endogenous RNA present in blood. This demonstrates that the RT-PCR RTG beads system when used in combination with low blood concentrations is able to overcome the effect of some RT-PCR inhibitors present. At higher blood concentration (0.1% v/v) even in the presence of added total RNA (200ng) RT-PCR inhibition was still apparent. Comparable results were obtained
when using Hela cell RNA with GAPDH and β-actin primer.

A system was therefore identified, in which the inhibitory effect was reduced i.e. the system was capable of generating a RT-PCR product from i) 0.1% (v/v) blood in the presence of added RNA and ii) endogenous RNA from blood < 0.025% (v/v). However, this amplification did appear to be sub-optimal.

Additional experiments were performed to investigate if the EDTA used to prevent the coagulation of blood was eliciting any inhibitory effect on the generation of direct RT-PCR products. EDTA is a known chelator of Mg²⁺ ions, and these ions are required as a cofactor for many DNA modifying enzymes including the MMLV-RT and Taq DNA polymerase used in the RT-PCR RTG beads. The amount of Mg²⁺ required is finely balanced and too much Mg²⁺ will result in enhanced stabilisation of double stranded DNA therefore making the duplex more resistant to denaturation which is an important initial step in PCR. Calculations of the EDTA present in the blood used in these experiments, appeared to be negligible. The amount of EDTA used in the anti-coagulant blood tubes is 6.1 mM. At a blood concentration of 0.1% (v/v), the amount of EDTA in the blood sample would be < 0.05 mM. This was considered insufficient to affect the efficiency of the RT-PCR process as the RT-PCR RTG beads contain 1.5 mM Mg²⁺ ions. However, for confirmation RT-PCR reactions were set-up in which additional amounts of MgCl₂ (1.5, 2.5 and 3.5 mM) was added to 0.1% (v/v) blood supplemented with total RNA. On completion the samples were subjected to gel electrophoresis and results indicated that no detectable decrease in the yield of the direct RT-PCR products was observed (data not shown). Therefore it was concluded that the EDTA present in the anti-coagulation system did not cause any significant inhibition to the generation of RT-PCR products.

As an attempt to further optimise the yield of the direct RT-PCR product a literature search was conducted to identify potential sources of RT-PCR inhibition. Inhibitors in blood which have been identified are either natural components of blood, mainly haem, hematin, haemoglobin, and immunoglobulin G or added anticoagulants such as EDTA and heparin (Al-Soud and Radstrom, 2001, J Clin Micro 39, 485-493). The interaction between these inhibitors and RNA or genomic DNA prevents the binding of the polymerases to the template. A component used to reduce inhibition of PCR is bovine serum albumin (BSA), which is able to bind to haem and prevent its inhibitory action on the polymerase. BSA has been shown to enhance nucleic acid amplification in the presence of blood-derived inhibitors such as haemoglobin and lactoferrin. BSA binds to haem (Fe²⁺), reducing the haem-derived inhibitory effect on the RT and DNA
polymerase enzymes. Therefore the addition of BSA may reduce the observed inhibition of the RT-PCR reaction. Based upon the studies of Comey et al, 1994; J.Forensic Sci 39, 1254-1269; Al-Soud & Radstrom, 2000; J Clin Micro 39, 485-493, BSA was added to the reaction mixture over a concentration ranging from 0.6% to 1.2% (w/v).

Experiments were performed in which the blood concentration was diluted with PBS from 0.025 - 0.02% (v/v) and the RT-PCR RTG bead mixture was supplemented with a BSA ranging from 0 - 1.2% (w/v). The method was also slightly modified to include a 15 minute SDS incubation at 37 °C, followed by a 5 minute room-temperature incubation with cyclodextrin. This ensures sufficient time for SDS to affect efficient cellular lysis and inhibit any RNase activity present. It will also facilitate the efficient sequestration and neutralization of SDS by cyclodextrin before the initiation of the RT-PCR reaction. Representative RT-PCR results are described in Figure 4.

Direct RT-PCR amplicons were generated in reactions initiated in the absence of any added total RNA (see Figure 4; lanes 4, 6, 8 and 10). Gel electrophoresis results indicate that the addition of BSA > 0.6% (w/v) supported the generation of a direct RT-PCR products. However, at elevated concentrations of BSA [1.2% (w/v)] a precipitation in the RT-PCR reaction mixture was noted. Comparable results were obtain, using the GAPDH and β-actin primer in the absence of added total RNA. A summary of the results are described in Table 1. Based upon these experiments it was concluded that optimal conditions for generating direct RT-PCR products using the RT-PCR RTG beads were 0.3% and 0.6% (w/v) BSA supplements in combination with 0.075% and 0.1% (v/v) blood respectively.

<table>
<thead>
<tr>
<th>Diluted Blood % (v/v)</th>
<th>Added BSA % (w/v)</th>
<th>0%</th>
<th>0.3%</th>
<th>0.6%</th>
<th>0.9%</th>
<th>1.2%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25%</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5%</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.75%</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.1%</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>0.2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Summary of direct RT-PCR results performed in the absence of added total RNA using RT-PCR RTG beads supplemented with increasing amounts of BSA. No visible amplicons observed (-), (+) and (++) indicate the successful generation of
increasing amounts of direct RT-PCR products. * precipitation was observed in the reaction tube.

**Formal Design of Experiment - Investigating the effect of added chemicals on the performance of direct RT-PCR using RT-PCR RTG beads**

As an attempt to further increase the amount of direct RT-PCR product using the RT-PCR RTG beads a number of nucleic acid amplification enhancers were identified. Their ability to increase RT-PCR product yield was investigated by performing a formal statistical Design of Experiment. To achieve this it was decided to use the combination of RT-PCR RTG beads supplemented with 0.6% (w/v) BSA with 0.1% (v/v) blood diluted with PBS a the model system from which to develop more enabling reaction mixtures.

Six chemicals considered to enhance PCR or RT-PCR reactions were selected for a formal Design of Experiment (DOE). These components included BSA, glycerol, Dimethyl sulfoxide (DMSO), Triton X-100, DTT and betaine.

Addition of BSA has been shown to enhance nucleic acid amplification in the presence of blood-derived inhibitors such as haemoglobin and lactoferrin (Comey et al, 1994; J.Forensic Sci 39, 1254-1269; Al-Soud & Radstrom, 2000; J Clin Micro 39, 485-493). BSA binds to haem (Fe²⁺), reducing the haem-derived inhibitory effect on reverse transcriptase and DNA polymerase enzymes.

DMSO is a polar solvent that dissolves both polar and non-polar compounds, therefore addition of DMSO promotes the solubilization of the reaction components in the presence of elevated BSA concentrations. DMSO is used as an enhancing agent and is included as part of a standard optimization of PCR amplifications (Varadaraj and Skinner, 1994; Gene, 140, 1 - 5). DMSO can also increase amplification specificity by destabilizing non-specific primer binding. Addition of 1-10% DMSO has been reported to increase the yield of PCR products by lowering annealing temperature thereby promoting primer binding and to disrupt regions of nucleic acid secondary structure [Simonovic et al, 2012; Arch. Bio. Sci (Belgrade) 64, 865-876].

The addition of glycerol (e.g. 5-20%) destabilizes nucleic acid duplexes and disrupts RNA secondary structure. The addition of glycerol to a PCR mixture is reported to increase the thermal stability of the polymerase and decreases the denaturation
temperature necessary for duplex strand separation (Cheng et al, 1994; PNAS 91 5695 - 5699).

Triton X-100 (and Brij 56 or 58) are non-ionic detergents, which can be used in PCR (e.g. up to 0.5%) to reduce DNA polymerases from associating or sticking to themselves or to the walls of the reaction tube. Triton X-100 can stabilize Taq DNA polymerase at elevated temperatures, aid solubilizing reaction components in the presence of elevated BSA concentrations and destabilizes nucleic acid secondary structures (Gelfand and White, 1990; Thermostable DNA polymerases pp 129-141 in PCR Protocols (Innis, Gelfand, Sinisky & White eds; Academic Press NY).

Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are reducing agents which have been reported to denature and prevent the formation of RNA secondary structure. DTT is used at ~5 mM in PCR buffers to help maintain the active conformational structure of the thermostable DNA polymerase (Van Pelt-Verkuil et al, 2008; Principles and Technical Aspects of PCR amplification). TCEP is a derivative of DTT that has been shown to increase RNA stabilisation and is reported to be more resistant to oxidation (Rhee and Burke, 2004; Anal Biochem 325, 137 - 143).

The addition of betaine (~1M) has been reported to improve nucleic acid amplification by reducing the formation of secondary structure in GC-rich regions (Rees et al, 1993 Biochem 32, 137-144 and Hencke et al, 1997; NAR 25, 3957-3958). DMSO is thought to aid nucleic acid amplification in a similar manner by interfering with hydrogen bond formation between the DNA duplex (Geiduschek and Herskovits, 1961; Arch Biochem Biophys, 95, 114-129).

A total of 48 experiments were performed during the DOE, investigating the effect of these chemicals on the performance of direct RT-PCR reactions. The experiments were performed using specific combination of these chemicals as defined in the DOE by the statistical SAS JMP software. These specific combinations were added to RT-PCR RTG beads to assess nucleic acid amplification. The DOE was designed to investigate the main effects of each individual chemical but also their 2nd and 3rd order interactions with the other chemicals.

An initial set of experiments were performed that identified an appropriate concentration of blood (0.1% v/v) and BSA (0.6% w/v) for use in DOE experiments. Additional experiments developed an efficient cellular lysis system using SDS and cyclodextrin and indicated that, EDTA (at the concentration used) did not exhibit any inhibitory effects on
the direct RT-PCR reaction. Individual experiments from the DOE were performed using a final blood concentration of 0.1% (v/v) and BSA 0.6% (w/v). All reactions were performed in triplicate.

Cells from EDTA anti-coagulated blood were lysed by the addition of SDS [final concentration of a 2% (w/v)] and incubated at room temperature for 15 min. The SDS was removed by the addition of freshly prepared cyclodextrin at a molar ratio of SDS:cyclodextrin 1:2 followed by a 5 minute room-temperature incubation. The mixture was then subjected to RT-PCR using RT-PCR RTG beads supplemented with combinations of chemicals as defined in the DOE (see Table 2). Direct RT-PCR reactions were performed as described earlier and RT-PCR products were subjected to agarose gel electrophoresis. A successful amplification was determined by the presence of a single PCR product of 291 bp for human β-globin (data not shown). To determine any improvement, the band intensity was compared to the 'standard' control reaction [0.1% blood using RT-PCR RTG beads supplemented with 0.6% (v/v) BSA]. Analysis was scored by visual inspection, with each reaction being given a score between -3 and +3 as a comparison to the 'standard', with +3 reflecting a significantly higher product yield and -3 a significantly lower product yield than the 'standard' reaction. Results are presented in Table 2.

The chemical combinations that generated a score > 7 were considered indicative of an improved direct RT-PCR product yield. These are summarised in Table 3. However to ensure that no alternative combinations were missed i.e. those that were not performed as part of the DOE (Table 2), statistical models were generated based upon the DOE results (see Figures 5, 6, 7 & 8). To indicate the efficacy of the DOE approach, the chemical composition of reaction 30 was equivalent to that of the control and during the "blind scoring", reaction 30 was scored the same as the control i.e. zero.
Table 2 - Chemical components in the Design of Experiment reactions. The presence and absence of chemicals in the DOE reactions are indicated by (1) and (0) respectively. For BSA (1) and (0) indicates 1% or 0.6% (w/v) respectively. Scores were determined by the visual inspection of RT-PCR product yield compared to that generated for the control reaction containing only BSA 0.6% (w/v). A score of +3 or -3 indicates a significantly greater or lower RT-PCR product yield compared to the control reaction.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Chemical combination</th>
<th>score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>BSA 0.6% (w/v), DMSO 5% (v/v) &amp; Glycerol 10% (v/v)</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v) &amp; Betaine (1 M)</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>BSA 1.0% (w/v) &amp; DMSO 5% (v/v)</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v), Triton 0.5% (v/v) &amp; Betaine (1 M)</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>BSA 1% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) &amp; DTT (10 mM)</td>
<td>9</td>
</tr>
<tr>
<td>18</td>
<td>BSA 0.6% (w/v) &amp; DMSO 5% (v/v)</td>
<td>9</td>
</tr>
<tr>
<td>21</td>
<td>BSA 1.0% (w/v), Glycerol 10% (v/v), Triton 0.5% (v/v) &amp; DTT (10 mM)</td>
<td>7</td>
</tr>
<tr>
<td>35</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) &amp; Triton 0.5% (v/v)</td>
<td>7</td>
</tr>
<tr>
<td>42</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Triton 0.5% (v/v) &amp; Betaine (1 M)</td>
<td>7</td>
</tr>
<tr>
<td>46</td>
<td>BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v), &amp; Triton 0.5% (v/v)</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3 - DOE chemical combinations that scored ≥ 7 (max possible = 9) compared to the control reactions during direct RT-PCR reactions.

Models based upon results from the Design of Experiment

Analysis of the DOE results was performed (see Table 2) to determine the optimum combination of components that would give the highest score (reflecting RT-PCR product yield based on band intensity). Using the SAS-JMP software and the associated Prediction Profiler software, statistical models were generated describing the mixture of components that gave the highest scores and therefore the highest RT-PCR product yield. The following models were identified.

1. BSA 1% (w/v), DMSO 5% (v/v), Triton X-100 0.5% (v/v) and betaine (1 M) (average score of 3.77) \( R^2 = 0.93 \). This model was performed as DOE reaction 42.
2. BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10 mM) (average score of 3.29) \( R^2 = 0.91 \). This model was performed as DOE reaction 13.
3. BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v) (average score of 3.26) \( R^2 = 0.63 \). This model was performed as DOE reaction 3.
4. BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) and DTT 4.1 mM (average score of 3.00; \( R^2 = 0.58 \)). This combination of chemicals were identified based upon the statistical model only and not as a direct result of the DOE. This combination of chemicals is identical to those used in reaction mixture 13 but the BSA and DTT are at different concentrations i.e. 0.6% (w/v) and 4.1 mM versus 1% (w/v) and 10 mM as defined in reaction 13 respectively.

The scores and \( R^2 \) values were generated using Prediction Profiler. \( R^2 \) values reflect the correlation between the actual experimentally derived scores and those predicted by the models. High \( R^2 \) values indicate good correlation and therefore the model exhibits a high predictive value. Graphs of actual versus predicted values to demonstrate \( R^2 \) (reflecting correlation), and results from Prediction Profiler are described in (see Figures 5, 6, 7 & 8).

To validate these models, confirmatory RT-PCR reactions were performed containing the predicted chemical components as described for each model. Direct RT-PCR was performed using 0.1% (v/v) blood, RT-PCR RTG beads, random hexamers and \( \beta \)-globin.
primers as described previously. The results are shown in Figures 9 and 10 and confirm the predictive performances of each model. The addition of the different chemicals to the appropriate direct RT-PCR reactions generated an increase in product yield compared to that of the control system.

**Model 1** - BSA 1% (w/v), DMSO 5% (v/v), Triton X-100 0.5% (v/v) and betaine (1 M)

**Model 2** - BSA 1% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT (10 mM)

**Model 3** - BSA 0.6% (w/v), DMSO 5% (v/v) and glycerol 10% (v/v)

**Model 4** - BSA 0.6% (w/v), DMSO 5% (v/v), glycerol 10% (v/v) and DTT 4mM

**Control** - BSA 0.6% (w/v)

Table 4 lists three groups of chemical combinations from the DOE results and statistical models. Group 1 contains at least BSA, DMSO and glycerol; Group 2 contains at least BSA and DMSO; and Group 3 contains at least BSA and glycerol.

<table>
<thead>
<tr>
<th>DOE Reaction</th>
<th>Chemical combination</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>BSA 0.6% (w/v), DMSO 5% (v/v) &amp; Glycerol 10% (v/v) – Model 3</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v), Triton (0.5% v/v) &amp; Betaine (1M)</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>BSA 1% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) &amp; DTT (10mM) – Model 2</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) &amp; Triton (0.5% v/v)</td>
<td>1</td>
</tr>
<tr>
<td>46</td>
<td>BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v), &amp; Triton (0.5% v/v)</td>
<td>1</td>
</tr>
<tr>
<td>n/a</td>
<td>BSA 0.6% (w/v), DMSO 5% (v/v), Glycerol 10% (v/v) and DTT 4.1mM – Model 4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v) &amp; Betaine (1 M)</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>BSA 1.0% (w/v) &amp; DMSO 5% (v/v)</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>BSA 0.6% (w/v) &amp; DMSO 5% (v/v)</td>
<td>2</td>
</tr>
<tr>
<td>42</td>
<td>BSA 1.0% (w/v), DMSO 5% (v/v), Triton 0.5% (v/v) &amp; Betaine (1 M) – Model 1</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>BSA 1.0% (w/v), Glycerol 10% (v/v), Triton 0.5% (v/v) &amp; DTT (10 mM)</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4 - Classification of the combination of chemical additives used to supplement and improve the performance of direct RT-PCR beads into specific Groups. The improved performance of these combinations were demonstrated either in the DOE or via validating statistical models. Group 1 contains at least BSA, DMSO and glycerol; Group 2 contains at least BSA and DMSO; and Group 3 contains at least BSA and glycerol.

**Direct amplification of RNA using RT-PCR RTG beads; Use of FTA sample collection cards**
Paper-based sample collection cards are a popular method of storing blood and other biological tissues due to their inherent properties of providing long term stability to nucleic acids and their ease of use in many molecular biology-based workflows. These cards are impregnated with various chemicals some of which are known to inhibit enzymatic reactions especially those involved in the amplification of nucleic acids such as PCR, RT-PCR etc. To overcome this challenge, several commercially available genetically engineered DNA polymerases have been developed, including Omniklentaq and Phusion. These enzymes have been engineered to amplify genomic DNA from complex biological samples. These samples often contain multiple PCR and nucleic acid amplification inhibitors. An alternative approach is exemplified by the generation of short tandem repeat (STR) profiles for Forensic human identification purposes. These approaches, utilise especially developed buffers and protocols that reduce inhibition effects. To date no comparable solutions have been reported for the direct amplification of RNA from chemically impregnated sample collection cards.

FTA sample collection cards (GE Healthcare) are examples of chemically-coated cards. Biological samples such as blood, saliva etc. are simply applied to the cards and the impregnated chemicals inactivate micro-organism, lyse cells, denature proteins and provide long term stability to nucleic acids. These cards are commonly used in direct nucleic acid amplification workflows in which no prior nucleic acid extraction and purification steps are needed. Small punches containing the biological sample are simply excised from the FTA cards and added directly to the amplification reaction. The resultant mixture of punch and reaction buffer is subjected to PCR.

RNA can be extracted from FTA cards using RNA extraction kits such as the Illustra RNAspin kit (GE Healthcare). In the associated workflow the cards are simply treated as tissue and homogenised using a 20 gauge needle. The resultant homogenate is then transferred to the RNAspin column for the subsequent purification of RNA according the manufacturer’s instructions. Based upon this finding, a study was performed to evaluate if RNA in a biological sample when applied to FTA cards could be amplified directly using the workflow and RT-PCR buffers as described earlier.

HeLa cells in PBS were applied to FTA sample collection cards. The cards were allowed to dry for 3 hours prior to the removal of a small section of the card which contained lysed HeLa cellular debris. This was accomplished using a Harris 1.2 mm diameter extraction punch. The small excised disc was subjected to the workflow previously described in which RNA was amplified directly from diluted blood. Briefly, the 1.2 mm punch was subject to a 15 minute SDS 2% (w/v) incubation at 37 °C, followed by a 5
minute room-temperature incubation with a SDS:cyclodextrin molar ratio of 1:2, followed by an incubation of 65°C for 10 min. The mixture plus punch was allowed to cool and added to RT-PCR RTG beads that were supplemented with i) β-actin RT-PCR primers, ii) the direct RT-PCR chemical additives described earlier (see Tables 3 & 4) and iii) random hexamers.

The thermocycle used was as follows; to initiate the RT reaction the punch and reagents were thoroughly mixed and incubated at 42 °C for 60 minutes, followed by PCR, involving 95°C for 3 minutes and the following thermocycle; 40 cycles at 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute with an extension of 72 °C for 5 minutes.

RT-PCR amplicons for β-actin of the correct 626 bp size were generated in all the RT-PCR reactions. The relative yield of each RT-PCR product was determined by the intensity of bands visualised on agarose gels. The yield of RT-PCR products associated with the reactions supplemented with the chemical additives were all greater than that derived from the control reaction supplemented with BSA (0.6% w/v) only. A representative agarose gel (Figure 11) showing the results from Model 1, Model 2, Model 3 and Model 4 compared to the control reaction is shown by way of illustration. Increased β-actin RT-PCR product yield was also observed from all RT-PCR RTG bead reactions supplemented with all the chemical additives described in Tables 3 & 4 (data not shown).

**Direct amplification of RNA by RT-PCR reactions in a liquid format; Use of diluted blood and HeLa cell applied to FTA sample collection cards**

The chemical combinations identified in both the actual DOE experiments and the statistical models are described in Tables 3 & 4. These chemical combinations were used to supplement liquid based direct RT-PCR reactions.

The reaction mixture consisted of EDTA anti-coagulated human blood diluted to 0.1% (v/v) with PBS. Briefly, the diluted blood was subject to a 15 minute 2% SDS (w/v) incubation at 37 °C, followed by a 5 minute room-temperature incubation with a 1:2 molar ratio of SDS:cyclodextrin, followed by an incubation of 65°C for 10 min. The mixture was allowed to cool and then subjected to direct RT-PCR as previously described using AffinityScript RT in combination with Taq DNA polymerase. The reaction was supplemented with i) β-globin specific RT-PCR primers; exon I forward 5'-GGT GAA CGT GGA TGA AGT TG-3' and exon III reverse 5'-AGC ACA
CAG ACC AGC ACG T- 3'; RT-PCR product size 291 bp ii) the direct RT-PCR chemical additives described in Table 3 & 4 and iii) random hexamers. The thermocycle used was as follows; to initiate the RT reaction the diluted blood and reagents were thoroughly mixed and incubated at 42 °C for 60 minutes, followed by the addition of Taq DNA polymerase, xIO PCR buffer etc. PCR involved heating for 95°C for 3 minutes and the following thermocycle; 40 cycles at 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute with an extension of 72 °C for 5 minutes. 

β-globin specific amplicons of the correct size were generated in all the RT-PCR reactions (Figure 12). A representative composite agarose gel showing the results from Model 1, Model 2, Model 3 and Model 4 compared to the control reaction is shown as way of illustration. The relative yield of each RT-PCR product was determined by the intensity of bands visualised on agarose gels and compared to that derived from control RT-PCR reaction containing 0.6% (w/v) BSA only. The yield of RT-PCR products associated with the chemical additives were all greater than that derived from the control reaction. Similar results were obtained when the other chemical additives also described in Tables 3 & 4 were used (data not shown).

Direct RT-PCR products were also generated from HeLa cells applied to FTA sample collection cards. HeLa cells in PBS were applied to FTA sample collection cards. The cards were allowed to dry for 3 hours prior to the removal of a small section of the card which contained lysed HeLa cellular debris. This excision was accomplished using a Harris 1.2 mm diameter extraction punch. The 1.2 mm punch was subject to a 15 minute SDS (2% w/v) incubation at 37 °C, followed by a 5 minute room-temperature incubation with a 1:2 molar ratio of cyclodextrin, followed by an incubation of 65°C for 10 min. The mixture plus punch was allowed to cool and then subjected to direct RT-PCR as previously described using AffinityScript RT in combination with Taq DNA polymerase. The reaction was supplemented with i) ; β-actin, exon 1 forward 5'-CCTCGCCCTTTGCGGATCC- 3' and exon 4 reverse 5'-GGATCTTTCATAGGTAGTCAGTC-3’ ; RT-PCR product size 626 bp ii) the direct RT-PCR chemical additives described in Tables 3 & 4 and iii) random hexamers.

The thermocycle used was as follows; to initiate the RT reaction the punch and reagents were thoroughly mixed and incubated at 42 °C for 60 minutes followed by the addition of Taq DNA polymerase, xIO PCR buffer etc. The PCR conditions consisted of; 95°C for 3 minutes and the following thermocycle; 40 cycles at 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute with an extension of 72 °C for 5 minutes.
RT-PCR amplicons for β-actin of the correct 626 bp size were generated in all the RT-PCR reactions. The relative yield of each RT-PCR product was determined by the intensity of bands visualised on agarose gels. The yield of RT-PCR products associated with the reactions supplemented with the chemical additives were all greater than that derived from the control reaction supplemented with BSA (0.6% w/v) only. A representative agarose gel (Figure 13) showing the results from Model 1 and Model 3 compared to the control reaction is shown as way of illustration. Increased β-actin RT-PCR product yield was also observed from all RT-PCR RTG bead reactions supplemented with all the chemical additives described in Tables 3 & 4 (data not shown).
CLAIMS

1. A method of amplifying an RNA molecule in a biological sample by reverse transcription PCR (RT-PCR), wherein the RT-PCR is carried out in a solution comprising:
   a. a polar aprotic solvent;
   b. a serum albumin; and optionally
   c. a non-ionic surfactant and/or a betaine.

2. A method according to claim 1, wherein the solution comprises:
   a. a polar aprotic solvent;
   b. a serum albumin; and
   c. a betaine.

3. A method according to claim 1, wherein the solution comprises:
   a. a polar aprotic solvent;
   b. a serum albumin;
   c. a non-ionic surfactant and
   d. a betaine.

4. A method according to claim 1 or claim 3, wherein the non-ionic surfactant is Triton X-100, Brij 56 or Brij 58.

5. A method according to any preceding claim, wherein the polar aprotic solvent is DMSO (dimethyl sulfoxide).

6. A method according to any preceding claim, wherein the serum albumin is bovine serum albumin (BSA).

7. A method according to any preceding claim, wherein the betaine is \( \text{N}_3\text{N}_3\text{N}_3\text{trimethylglycine} \).
8. A method according to claim 1, wherein the solution comprises:

(a) DMSO at a concentration in the range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to 1.2% (w/v); Triton X-100 at a concentration in the range 0.05 to 1% (v/v); and N,N,N-trimethylglycine at a concentration in the range 0.05 to 2M;

(b) DMSO at a concentration in the range 0.05 to 10% (v/v), BSA at a concentration in the range 0.05 to 1.2% (w/v); or

(c) DMSO at a concentration in the range 0.05 to 10% (v/v); BSA at a concentration in the range 0.05 to 1.2% (w/v); and N,N,N-trimethylglycine at a concentration in the range 0.05 to 2M.

9. A method according to any preceding claim, wherein the solution further comprises:

a. a reverse transcriptase;

b. a DNA polymerase;

c. a deoxyribonucleotide triphosphate (dNTP); and

d. at least one primer.

10. A method according to any preceding claim, wherein the biological sample has not undergone treatment with a DNase prior to the RT-PCR reaction.

11. A method according to any preceding claim, wherein the biological sample has not undergone an RNA purification step prior to the RT-PCR reaction.

12. A method according to any preceding claim, further comprising subjecting the biological sample to a lysis step and a detergent neutralization step prior to contacting the biological sample with the solution.

13. A method according to claim 12, wherein the lysis step is performed by contacting the biological sample with a detergent, preferably sodium dodecyl sulphate (SDS), and the detergent neutralization step is performed by contacting the sample with a cyclodextran.
14. A method of according to any preceding claim, wherein the biological sample comprises a cellular sample selected from the group consisting of blood, serum, semen, cerebral spinal fluid, synovial fluid, lymphatic fluid, saliva, buccal, cervical cell, vaginal cell, urine, faeces, hair, skin, muscle, and cells grown in culture.

15. A method according to any preceding claim, wherein the biological sample is derived from a virus, a eukaryotic organism or a prokaryotic organism.

16. A method according to claim 14, wherein the biological sample is a blood sample, optionally where the blood has been treated with an anti-coagulant.

17. A method according to any preceding claim, wherein the RNA is immobilised on a solid support and the solid support is contacted with the solution.

18. A method according to claim 17, comprising:
   i) contacting a solid support with a biological sample containing the RNA;
   ii) transferring the solid support, or a portion thereof, to a reaction vessel optionally after a washing step; and
   iii) performing the RT-PCR reaction in the reaction vessel in the solution in the presence of the solid support.

19. A method according to claim 17 or claim 18, wherein the solid support:
   a) is fibrous, optionally comprising a cellulose fibre material or a glass fibre or glass microfiber material; or
   b) comprises a porous polymer, optionally a porous membrane material such as polyester, polyether sulfone (PES), polyamide (Nylon), polypropylene, polytetrafluoroethylene (PTFE), polycarbonate, cellulose nitrate, cellulose acetate or alignate; or
   c) comprises aluminium oxide.
20. A method according to any one of claims 17 to 19, wherein a lysis reagent is embedded on the solid support.

21. A method according to any one of claims 17 to 20, wherein the solid support is impregnated with one or more of (i) a weak base; (ii) a chelating agent; (iii) an anionic surfactant and (iv) a chaotropic agent such as guanidium thiocyanate.

22. A dried reagent composition for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), the composition comprising a sequestering reagent; a polymerase; a deoxyribonucleotide triphosphate (dNTP); a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant.

23. A dried reagent composition according to claim 22, comprising a sequestering reagent; a polymerase; a deoxyribonucleotide triphosphate (dNTP); a serum albumin; a betaine; a non-ionic surfactant; and optionally a polar aprotic solvent.

24. A dried reagent composition according to claim 22 or claim 23, wherein the sequestering agent is cyclodextrin.

25. A dried reagent composition according to any one of claims 22 to 24, wherein the serum albumin is bovine serum albumin (BSA) and the polar aprotic solvent, if present, is DMSO (dimethyl sulfoxide).

26. A dried reagent composition according to any one of claims 22 to 25, wherein the non-ionic surfactant is Triton X-100, Brij 56 or Brij 58, and/or the betaine is N,N,N-trimethylglycine.

27. A dried reagent composition according to any one of claims 22 to 26, comprising the polar aprotic solvent.

28. A method for producing a dried reagent composition as defined in any one of
claims 22 to 27 for amplifying an RNA molecule by reverse transcription PCR (RT-PCR), comprising the steps:

a. combining a sequestering reagent; a polymerase; a dNTP; a serum albumin; a betaine; and optionally a polar aprotic solvent and/or a non-ionic surfactant to provide a mixture thereof; and

b. drying the mixture.

29. A method according to claim 28, wherein the drying step is achieved by lyophilizing.

30. A method according to claim 28 or claim 29, comprising freezing said composition prior to the drying step.

31. A method of amplifying an RNA molecule comprising the steps:

i) incubating the RNA molecule and the dried reagent composition of any one of claims 22 to 27 in a solution; and

ii) performing a reverse transcription PCR (RT-PCR) reaction.

32. A method according to claim 31, wherein the solution comprises DMSO that is not derived from the dried reagent composition.

33. A method according to claim 32, wherein the dried composition does not comprise DMSO.
FIG. 5A
## Sorted Parameter Estimates

| Term                                      | Estimate | Std Error | t Ratio | Prob>|t| |
|-------------------------------------------|----------|-----------|---------|------|
| DMSO                                      | 1.3547261| 0.187842  | 7.21    | <.0001*|
| Glycerol                                  | 0.9991793| 0.189003  | 5.29    | <.0001*|
| DTT                                       | -0.927955| 0.186637  | 4.97    | <.0001*|
| (BSA-0.5)*(Glycerol-0.5)*(DTT-0.47917)    | 3.2178926| 0.79525   | 4.05    | 0.0006*|
| (BSA-0.5)*(DTT-0.47917)*(Betaine-0.52083)| -2.932082| 0.803168  | -3.65   | 0.0016*|
| (DMS0-0.5)*(Glycerol-0.5)*(DTT-0.47917)   | 2.6843308| 0.792817  | 3.39    | 0.0029*|
| (DMS0-0.5)*(DTT-0.47917)                  | -1.235115| 0.382396  | -3.23   | 0.0042*|
| (BSA-0.5)*(Triton-0.47917)*(DTT-0.47917)| -2.481461| 0.80993   | -3.06   | 0.0061*|
| (Glycerol-0.5)*(Betaine-0.52083)          | -1.127959| 0.385537  | -2.93   | 0.0084*|
| (DMS0-0.5)*(Triton-0.47917)*(DTT-0.47917)| -2.155344| 0.80687   | -2.67   | 0.0147*|
| (DMS0-0.5)*(Triton-0.47917)               | -1.014366| 0.39872   | -2.54   | 0.0193*|
| (Glycerol-0.5)*(Triton-0.47917)*(Betaine-0.52083)| -1.967245| 0.795902  | -2.47   | 0.0226*|
| (Triton-0.47917)*(DTT-0.47917)*(Betaine-0.52083)| -1.725192| 0.764016  | -2.26   | 0.0353*|
| (DMS0-0.5)*(Betaine-0.52083)              | 0.7504841| 0.396978  | 1.89    | 0.0733 |
| (Glycerol-0.5)*(Triton-0.47917)           | 0.6624075| 0.378138  | 1.75    | 0.0951 |
| (BSA-0.5)*(Triton-0.47917)                | -0.67361 | 0.387118  | -1.74   | 0.0972 |
| (DMS0-0.5)*(Glycerol-0.5)                 | -0.643264| 0.399696  | -1.61   | 0.1232 |
| (Triton-0.47917)*(Betaine-0.52083)        | 0.5385503| 0.378176  | 1.42    | 0.1698 |
| BSA                                       | 0.2255069| 0.18581   | 1.21    | 0.2390 |
| Triton                                    | -0.202669| 0.188377  | -1.08   | 0.2948 |
| (BSA-0.5)*(Betaine-0.52083)               | -0.42112 | 0.396264  | -1.06   | 0.3006 |
| Betaine                                   | -0.176577| 0.19017   | -0.93   | 0.3642 |
| (BSA-0.5)*(DTT-0.47917)                   | -0.272826| 0.38625   | -0.71   | 0.4881 |
| (Glycerol-0.5)*(DTT-0.47917)              | -0.237972| 0.37945   | -0.63   | 0.5377 |
| (Triton-0.47917)*(DTT-0.47917)            | -0.145813| 0.37724   | -0.39   | 0.7032 |
| (DTT-0.47917)*(Betaine-0.52083)           | -0.118991| 0.381692  | -0.31   | 0.7585 |
| (BSA-0.5)*(Glycerol-0.5)                  | -0.062413| 0.389541  | -0.16   | 0.8743 |

**FIG. 5B**
FIG. 5C
Response Score Average

Actual by Predicted Plot

Score Average Actual

Score Average Predicted P<.0001
RSq=0.91 RMSE=0.6592

Summary of Fit
RSquare 0.912151
RSquare Adj 0.803386
Root Mean Square Error 0.659173
Mean of Response 0.6875
Observations (or Sum Wgts) 48

Analysis of Variance

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<th>F Ratio</th>
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<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

Residual by Predicted Plot

FIG. 6A

SUBSTITUTE SHEET (RULE 26)
## Sort Parameter Estimates

| Term                                      | Estimate | Std Error | t Ratio | Prob>|t| |
|-------------------------------------------|----------|-----------|---------|-----|---|
| DMSO                                      | 1.3586834| 0.199008  | 6.83    | <.0001* |
| Glycerol                                  | 0.995585 | 0.200241  | 4.97    | <.0001* |
| DTT                                       | -0.922537| 0.197771  | -4.66   | 0.0001* |
| (BSA-0.5)*(Glycerol-0.5)*(DTT-0.47917)    | 2.8638754| 0.818885  | 3.50    | 0.0021* |
| (BSA-0.5)*(DTT-0.47917)*(Betaine-0.52083)| -2.729562| 0.843364  | -3.24   | 0.0040* |
| (DMSO-0.5)*(DTT-0.47917)                 | -1.253834| 0.405018  | -3.10   | 0.0055* |
| (DMSO-0.5)*(Glycerol-0.5)*(DTT-0.47917)  | 2.4960827| 0.833349  | 3.00    | 0.0069* |
| (DMSO-0.5)*(Triton-0.47917)              | -1.143757| 0.416178  | -2.75   | 0.0120* |
| (Glycerol-0.5)*(Betaine-0.52083)         | -1.099048| 0.40816   | -2.69   | 0.0136* |
| (BSA-0.5)*(Triton-0.47917)*(DTT-0.47917)| -2.141869| 0.83676   | -2.56   | 0.0183* |
| (DMSO-0.5)*(Triton-0.47917)*(DTT-0.47917)| -2.061349| 0.853265  | -2.42   | 0.0249* |
| (Glycerol-0.5)*(Triton-0.47917)*(Betaine-0.52083)| -1.717675| 0.831589  | -2.07   | 0.0514 |
| (Triton-0.47917)*(DTT-0.47917)*(Betaine-0.52083)| -1.630126| 0.80773   | -2.02   | 0.0565 |
| (BSA-0.5)*(Triton-0.47917)               | -0.785031| 0.405375  | -1.94   | 0.0664 |
| (Glycerol-0.5)*(Triton-0.47917)          | 0.6725359| 0.400603  | 1.68    | 0.1080 |
| (Triton-0.47917)*(Betaine-0.52083)       | 0.5563057| 0.400559  | 1.39    | 0.1794 |
| (DMSO-0.5)*(Glycerol-0.5)                | -0.525843| 0.418339  | -1.26   | 0.2226 |
| BSA                                       | 0.2441206| 0.196592  | 1.24    | 0.2280 |
| Triton                                    | -0.235942| 0.198715  | -1.19   | 0.2484 |
| (BSA-0.5)*(Betaine-0.52083)              | -0.470739| 0.418925  | -1.12   | 0.2738 |
| Betaine                                   | -0.183701| 0.201448  | -0.91   | 0.3722 |
| (Triton-0.47917)*(DTT-0.47917)           | -0.186815| 0.39903   | -0.47   | 0.6445 |
| (BSA-0.5)*(DTT-0.47917)                  | -0.187499| 0.406434  | -0.46   | 0.6493 |
| (Glycerol-0.5)*(DTT-0.47917)             | -0.181153| 0.40077   | -0.45   | 0.6559 |
| (DTT-0.47917)*(Betaine-0.52083)          | -0.102043| 0.404296  | -0.25   | 0.8032 |
| (BSA-0.5)*(Glycerol-0.5)                 | 0.0089014| 0.410784  | 0.02    | 0.9829 |

**FIG. 6B**
Response Score Average

Actual by Predicted Plot

Score Average Predicted P<.0001
RSq=0.63 RMSE=1.0356

Summary of Fit
RSquare 0.628263
RSquare Adj 0.514676
Root Mean Square Error 1.035638
Mean of Response 0.6875
Observations (or Sum Wgts) 48

Analysis of Variance

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<tr>
<th>Source</th>
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<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
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<td></td>
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<tr>
<td>C. Total</td>
<td>47</td>
<td>103.86806</td>
<td></td>
<td>&lt;.0001*</td>
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Residual by Predicted Plot

FIG. 7A

SUBSTITUTE SHEET (RULE 26)
### Sorted Parameter Estimates

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<th>Term</th>
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<td>4.29</td>
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<td>Glycerol</td>
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<td>0.302569</td>
<td>3.80</td>
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<td>DTT</td>
<td>-0.8670077</td>
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<td>(DMSO-0.5)*</td>
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<td>(Glycerol-0.5)*</td>
<td>2.8669879</td>
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<td>(BSA+0.5)*</td>
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<td>Triton</td>
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<td>BSA</td>
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### Prediction Profiler

**FIG. 7B**

- **Desirability**
- **DMSO**
- **Glycerol**
- **BSA**
- **Triton**

---

SUBSTITUTE SHEET (RULE 26)
Neural
Validation: Random Holdback

Model NTanH(3)

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</table>

Actual by Predicted Plot

![Graphs showing actual vs predicted scores for training and validation sets.]

Prediction Profiler

![Bar chart showing prediction profiler for desirability and score average.]

FIG. 8

SUBSTITUTE SHEET (RULE 26)
FIG. 11

FIG. 12
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2016.01)
CPC - C12Q 1/68 (2016.02)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12P 19/30, 19/34; C12Q 1/00, 1/68 (2016.01)
CPC - C12Q 1/00, 1/68, 1/6844, 1/6846 (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
U.S. Classes - 435/4, 6.1, 6.12, 91.2 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, PubMed, Google.
Search terms used: reverse transcription PCR amplify rna polar aprotic solvent serum albumin surfactant betaine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search
11 March 2016

Date of mailing of the international search report
25 MAR 2016

Name and mailing address of the ISA:
Mail Stop PCT, Attn: ISA-US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450
Facsimile No. 571-273-3300

Authorized officer
Blaine R. Copenheaver
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)
INTERNATIONAL SEARCH REPORT

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.: 5-7, 9-21, 25-33 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying 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.: