(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 October 2006 (12.10.2006)

PCT

(10) International Publication Number
WO 2006/107686 A1

(51) International Patent Classification:
C12P 19/34 (2006.01) *C07H 21/04* (2006.01)
C12N 9/00 (2006.01)

(21) International Application Number:
PCT/US2006/011554

(22) International Filing Date: 30 March 2006 (30.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/096,775 1 April 2005 (01.04.2005) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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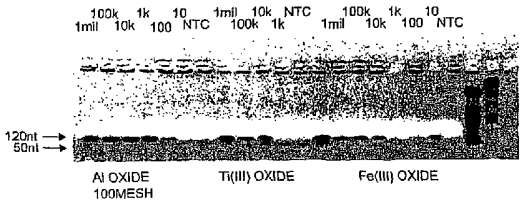
(54) Title: METHODS, COMPOSITIONS, AND KITS FOR DETECTING NUCLEIC ACIDS IN A SINGLE VESSEL



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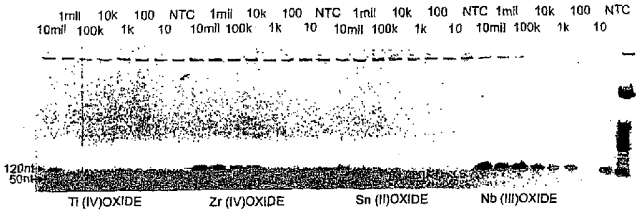

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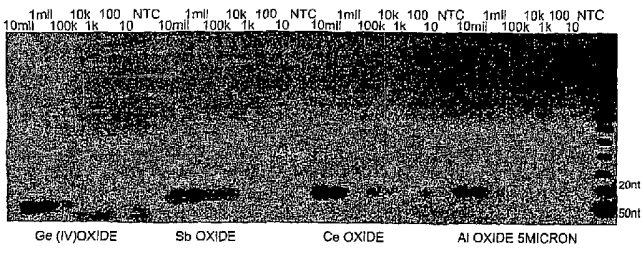

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(57) Abstract: The invention encompasses processes, compositions, and kits for isolating and detecting nucleic acids from samples using a metal oxide coated onto a vessel. The nucleic acids can be processed and detected within this vessel.

WO 2006/107686 A1

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS, COMPOSITIONS, AND KITS FOR DETECTING NUCLEIC ACIDS IN A SINGLE VESSEL

This application claims the benefit of priority of U.S. Patent Application No. 11/096,775, filed April 1, 2005.

Background of the Invention

Field of the Invention

The present invention relates to purifying, concentrating, and detecting nucleic acids in complex biological samples.

Related Art

In the field of nucleic acid detection, genetic analysis can involve the extraction and purification of nucleic acids from cells or tissues, amplification of the target nucleic acids, and detection of the sequence of interest.

There are many different ways to release genetic material from an organism. High temperatures or pressures can cause cells to lyse. Enzymatic approaches such as lysozyme and protease treatments aid removing critical membrane structures in cells and tissue to release cellular contents. Alkaline treatment and chaotropic salts can affect osmotic pressure outside the cells causing them to burst open.

Once the cells have been opened, the nucleic acid of the organism must be in a form suitable for detection. Some methods for preparing RNA or DNA are laborious using hazardous reagents to obtain purified substrates. For example, organic solvents such as phenol and chloroform can be used to lyse cells and to remove proteins and cell debris in the organic phase, leaving the nucleic acids in the aqueous phase. The RNA or DNA can be precipitated with an alcohol or spooled onto glass rods to concentrate the nucleic acid for downstream processing.

A crude purification of nucleic acids can also be done by a "salting out" technique that precipitates proteins with a high concentration of LiCl, leaving the nucleic acids in the solution. Alternatively, purifying nucleic acids from cell debris can be accomplished by a using a CsCl gradient to purify nucleic acids based on their size. This method requires a high concentration of the nucleic acid substrate and expensive equipment, such as an ultracentrifuge.

Another technique employed for purifying nucleic acids employs an electropositive, hydrophilic solid matrix resin such as Silicon, Boron, or Aluminum to specifically bind

nucleic acids in high salt and/or specific pH conditions. The DNA or RNA is eluted from the resins by changing the salt concentrations and pH.

For example, Boom et al. (U.S. Patent No. 5,234,809) disclose the use of a nucleic acid binding solid phase of silica particles capable of binding the nucleic acid in the presence of a chaotropic substance. Similarly, Woodard et al. (U.S. Patent Nos. 5,405,951, 5,438,129; and 5,438,127) disclose the use of binding matrixes having a hydrophilic surface, such as nitrocellulose, celite diatoms, silica polymers, glass fibers, magnesium silicates, silicone nitrogen compounds (e.g., SIN_4), aluminum silicates, and silica dioxide. The purified nucleic acid can be eluted with a mild buffer solution.

Efficient elution of the substrates from the solid support has been investigated (U.S. Patent No. 5,523,392; 5,525,319; and 5,503,816). To date, glass and silica have been employed in different formats such as slurries precipitated by centrifugation, glass beads in a chromatography format, or silica coated iron oxides for a magnetic precipitation of nucleic acids extracted from solution.

Another method for purifying nucleic acids employs anion exchange resins. As described in Patent Nos. 5,990,301; 6,020,186; 6,277,648B1, 6,609,618B2, and 6,674,371B1, resins coated with anion exchangers such as DEAE and DMAE bind specifically to nucleic acids in lysed biological samples. The binding of nucleic acids to this resin is dependent on the pH/ salt concentration under which proteins and cell debris do not bind. The DNA or RNA can be eluted with a change in the salt and pH conditions.

Anion exchange chemistries have also been used in different formats to bind nucleic acids in solution. A widely use format is a chromatographic based system in which a sample flows through a column containing an anion exchange resin. A major problem with this format is that it cannot handle large particulates and requires removal of the cell debris by centrifugation. Another permutation of this technology is to coat iron oxide with silica and an anion exchanger, such as DEA, to bind the nucleic acids in biological sample, and then to precipitate the complex with a magnet (U.S. Patent Nos. 6,368,800B1; 6,673,631; 6,027,945; 6,218,531B1; and 5,587,286). Recovery of the eluted DNA from anion exchangers yields at most 60% of the nucleic acids bound to resins.

The need for detecting few target molecules in large volumes that contain high levels of extraneous background DNA from complex biological samples has required new approaches to extract, purify, and concentrate nucleic acids for detection. Newer strategies involve higher affinity solid matrices for binding specifically to DNA or RNA.

These nucleic acid binding matrices include metal oxides such as magnesium, calcium, titanium, manganese, cobalt, nickel, zinc, yttrium, lanthanum, aluminum, iron, zirconium, and hafnium (EP 0391608A2). Metal oxides have a wide range of affinity for binding and extracting nucleic acids. Iron oxides appear to bind DNA or RNA with a relatively high affinity and have the property of reversible binding of nucleic acids under phosphate buffer conditions (U.S. Patent Appln. No. 2002/0068821 A1). Hydrated zirconium oxide beads, hafnium oxide beads, and aluminum oxide beads have been shown to have the ability to bind nucleic acids, which can then be easily eluted (EP 0897978 A2).

Metal oxides, such as zirconium, iron, cobalt, and hafnium oxides, bound to DNA can allow hybridization of probes to the bound nucleic acids (EP 0391698 A2). Moreover, aluminum oxide bound to DNA can permit hybridization of primers and amplification of the target by PCR (U.S. Patent No 6,291,166). In this patent, the unlabeled amplification products were detected by gel electrophoresis after removal from the PCR tube.

The rapid detection of pathogens in biological samples has become an important issue in clinical and environmental diagnostics. In order to detect low copy numbers of pathogens, further advances in sample preparation and detection methods must occur. Extremely efficient lysis methods for releasing the nucleic acids from the target organism could improve the sensitivity and accuracy of early detection. Likewise, the ability to perform preparation and detection steps in the same vessel could improve sensitivity and accuracy.

Thus, there exists a need in the art for new methods of preparing and detecting nucleic acids. The present invention fulfills this need.

Brief Summary of the Invention

The invention encompasses a method for the detection of a nucleic acid comprising attaching a nucleic acid molecule to a metal oxide coated onto a vessel; amplifying, in the vessel, a fragment of the nucleic acid molecule to generate an amplified product; and detecting, in the vessel, the amplified product. The invention further encompasses a method for the detection of a microorganism comprising lysing a microorganism to release a nucleic acid molecule; contacting the nucleic acid molecule with a vessel coated with at least one metal oxide; allowing the nucleic acid molecule to attach to the metal oxide; amplifying, in the vessel, a fragment of the nucleic acid molecule attached

to the metal oxide to generate an amplified product; and detecting, in the vessel, the amplified product.

In one embodiment, the microorganism can be lysed with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, and an alkaline solution; with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, a detergent, and an alkaline solution; or with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, and a NaCl solution of a concentration greater than 250mM.

Reagents that cleave peptidoglycan bonds include lysozyme, lysostaphin, or mutanolysin.

Proteases include proteinase K, pronase E, achromopeptidase, proteinase R, proteinase T, subtilisin DY, an alkaline serine protease from *Streptomyces griseus* or *Bacillus lichenformis*, dispase, subtilisin Calsberg, subtilopeptidase A, and thermolysin.

Alkaline solutions include 50mM-500mM KOH or NaOH.

Detergents include t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), and sorbitan monolaurate (Span 20).

In another embodiment, the microorganism is lysed with a chaotropic salt or a cationic surfactant. Chaotropic salts include guanidinium salts, such as guanidine hydrochloride, guanidine thiocyanate, guanidinium chloride, guanidinium hydrochloride, and guanidinium isothiocyanate.

Cationic surfactants include cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), tetradecetyltrimethylammonium bromide (TTAB), tetradecetyltrimethylammonium chloride (TTACl), dodecyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DTACl), dodecylethyldimethylammonium chloride (DEDTAB), decyltrimethylammonium bromide (D10TAB), and dodecyltripheylphosphonium bromide (DTPB).

Metal oxides include an oxide of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum, chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, and iron.

Vessels include a tube, column, plate, and well. In a preferred embodiment, the vessel is a PCR tube.

In one embodiment, the attached nucleic acid molecule is washed with a buffer that comprises t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), sorbitan monolaurate (Span 20), methanol, ethanol, or isopropenol. In a preferred embodiment, the buffer comprises at least 5% w/w ethanol and at least 0.5% w/w polyoxyethylenesorbitan monolaurate (Tween-20).

In one embodiment, the amplified product is detected by hybridization to a probe. Probes include a labeled DNA, PNA, RNA, o-methyl RNA, LNA, or modified nucleotide probe. The probe can be fluorescently labeled.

In another embodiment, the amplified product is detected with an intercalating dye. In another embodiment, the nucleic acid molecule can be amplified with a fluorescently labeled primer.

In one embodiment, the nucleic acid molecule is amplified by the polymerase chain reaction (PCR). In one embodiment, the amplified product is detected during amplification. In another embodiment, the amplified product is detected after amplification.

The invention encompasses a kit for detecting nucleic acids in a biological sample comprising a vessel coated with at least one metal oxide; instructions for binding a nucleic acid molecule to the metal oxide; and instructions for amplifying and detecting, in the vessel, a nucleic acid molecule bound to the metal oxide.

In another embodiment, the invention encompasses a kit for detecting nucleic acids in biological sample comprising a vessel coated with at least one metal oxide and instructions for lysing a microorganism to release a nucleic acid molecule, contacting the nucleic acid molecule with a vessel coated with at least one metal oxide, allowing the nucleic acid molecule to attach to the metal oxide; amplifying, in the vessel, a fragment of the nucleic acid molecule attached to the metal oxide to generate an amplified product, and detecting, in the vessel, the amplified product.

The kit can include primers for amplifying a nucleic acid molecule bound to the metal oxide. The kit can also include a fluorescent probe for detecting a nucleic acid molecule bound to the metal oxide.

The kit can include any of a lysozyme, a protease, a nonionic detergent, an alkaline solution and a NaCl solution of a concentration greater than 250mM.

Brief Description of the Drawings

The invention is more fully understood with reference to the drawings, in which:

FIG. 1A-C depict ethidium bromide stained agarose gels of PCR products amplified directly from *Listeria* genomic DNA bound to a metal oxide.

FIG. 2A and B are graphs of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide pretreated with a basic solution verses no pretreatment prior to adding the genomic DNA.

FIG. 3 is a graph of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide using 800mM guanidine to lyse open gram (+) micro-organisms.

FIG. 4 is a graph of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide using lysosyme, protease, a detergent, and an alkaline solution to lyse open gram(+) micro-organisms to increase the sensitivity of detection limits.

FIG. 5 is a graph of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide using lysosyme, detergent, and an alkaline solution to lyse open gram(+) micro-organisms to increase the sensitivity of detection limits.

FIG. 6 is a graph of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide using 800mM guanidine to lyse open gram (-) micro-organisms.

FIG. 7 is a graph of real time PCR detection of a specific nucleic acid bound to PCR tubes coated with aluminum oxide using lysosyme, protease, a detergent, and an alkaline solution to lyse open gram(-) micro-organisms to increase the sensitivity of detection limits.

FIG. 8 is a graph showing the effect of lysozyme on lysis, capture, and detection of micro-organisms in a single vessel apparatus.

FIG. 9 is a graph showing the effect of protease on lysis, capture, and detection of micro-organisms in a single vessel apparatus.

FIG. 10 is a graph showing concentrations of primers and probes in the real-time PCR reaction for the detection of low copies of the specific nucleic acid.

FIG. 11 is a graph showing the effect of high concentration of primers and probes in the real-time PCR reaction to improve the detection of low copies of the specific nucleic acid.

FIG. 12 is a graph showing an improvement of detection of low copies of bacteria using Line-PCR which has the forward primer in higher concentration than the reverse primer.

FIG. 13 is a graph showing an improvement of detection of low copies of bacteria using Line-PCR which has the reverse primer in higher concentration than the forward primer.

FIG. 14 A and B are graphs showing that NaCl can be substituted for NaOH for bacterial lysis and binding of released DNA onto aluminum oxide.

FIG. 15 is a graph showing the effect of NaCl concentration on bacterial lysis and binding of released DNA onto aluminum oxide.

Detailed Description of the Invention

The invention relates to novel methods, compositions, and kits for purifying and detecting nucleic acids. The invention encompasses preparing nucleic acids from complex biological samples, capturing the nucleic acids, removing inhibitors from the sample, and specifically amplifying and detecting the nucleic acids in a single vessel. The detection can be performed during ("real time detection") or after ("end point detection") the amplification reaction. The invention increases accuracy and sensitivity of detection of low copies of pathogens in large volumes while decreasing time and consumable cost to diagnostic laboratories.

METHODS

In one embodiment, the invention encompasses a process to release, extract, purify, and concentrate low copy numbers of nucleic acid substrates from a complex biological sample for the amplification and detection of a specific target.

In a preferred embodiment, the invention includes an efficient lysis method to extract the genetic material from the cells or tissues of interest. In one embodiment, the invention provides a novel protocol to lyse a biological sample and then capture, concentrate, and detect the genetic material on a solid matrix within a single vessel. The lysis protocol comprises a lysozyme treatment, a protease treatment, a detergent treatment, and a high ionic or alkaline condition to lyse open the cells.

In one embodiment, the lysis protocol comprises a lysozyme treatment, a protease treatment, and a high NaCl condition to lyse open the cells.

In another embodiment, the invention includes a high affinity solid support to strongly and specifically bind the nucleic acids in the sample, but not to other cellular components. Preferably, metal oxides are used that have the property of high affinity binding to nucleic acids since they have a strong positive charge to attract the

negatively charged phosphodiester backbone of RNA and DNA. The metal oxide binds the nucleic acids in the sample.

Elution of the substrate from the solid support is not required for direct detection of a specific target, since enzymatic and hybridization assays can occur on the target bound to the solid matrix. Furthermore, metal oxides have the advantage of binding RNA or DNA in a wide range of pH and salt concentration which is different from other solid supports consisting of silica or anion exchange resins. The nucleic acids bound to the metal oxide can be detected, in the same vessel, by an amplification of the genetic material in the presence of a labeled probe and a buffer that prevents further nucleic acids from binding to the solid matrix.

NUCLEIC ACIDS

Nucleic acids can be from any organism including animals and plants. Preferably, the nucleic acid is DNA, although RNA, especially rRNA or mRNA, can also be used. Preferably, the nucleic acid is from a microorganism. Preferred microorganisms are bacteria, fungi, yeasts, and viruses.

Preferred bacteria include *E. coli* (*e.g., E. coli* O157:H7), *Listeria (e.g., L. monocytogenes), Salmonella, Campylobacter, Legionella, (e.g., L. pneumophila) Bacillus (e.g., B. anthracis), Mycobacterium (e.g., M. tuberculosis), Mycoplasma, Clostridium, Chlamydia, Proteus, Enterococcus, Enterobacter, Pseudomonas, Neisseria, Staphylococcus (e.g., S. aureus), Streptococcus, Vibrio, Shigella, Helicobacter (e.g., H. pylori), Clostridium, Yersinia, Haemophilus, Pneumococcus,* and *Seratia.* Preferred viruses include human immunodeficiency viruses (*e.g.,* HIV-1), hepatitis viruses (*e.g.*, Hepatitis B virus), herpesviruses, rhinoviruses, papillomaviruses, and adenoviruses.

Particularly preferred bacteria are pathogenic bacteria, especially those found in food products, such as *E. coli* O157:H7, *Listeria*, *Salmonella,* and *Campylobacter.*

SAMPLES

Nucleic acids can be present in a variety of samples. Such sample types include biological samples such as blood, buccal swabs, mouth wash rinses, spinal fluid, hair follicles, mouse tails, nail follicles, tissue sections, urine, feces, and cervical fluids. The samples can also be food samples, water samples, environmental sources, forensic sources, and amplification reactions. The samples can be swabs in a food

processing plant. In a preferred embodiment, the sample is a food sample, such as a meat, poultry, or dairy product.

The samples can be manipulated prior to use in the invention, such as by filtering, purifying, disrupting tissues or cells, homogenizing tissues, centrifuging, precipitating, and/or adding reagents to the sample. The sample can be an amplified product. Samples can also be cultures, such as tissue culture samples or fermented broths. For example, samples can be cultured in an appropriate growth medium for the microorganism of interest to increase the numbers of microorganisms for detection. In one embodiment, inhibitors of other microorganisms can be added to select for increases in a particular microorganism or set of microorganisms in the culture. For example, specific antibiotics or anti-mycotics can be added to the culture to prevent the growth of susceptible microorganisms. Various dyes (such as crystal violet and acriflavin), salts (such as sodium chloride and lithium chloride), and detergents (such as sodium dodecyl sulfate) can also be used as selective agents. Increased or decreased temperature and oxygen tension can also produce selective growth conditions.

SAMPLE LYSIS

Samples can be lysed to release the target nucleic acid of interest from the organism. Lysis can be performed prior to contact with a nucleic acid binding matrix. Alternatively, lysis can be performed in a vessel together with a nucleic acid binding matrix.

Many different lysis procedures can be used. Procedures can vary depending on the strength of the outer membranes of the organism of interest. In organisms that have highly resistant outer membranes, mechanical methods, such as a homogenizer, bead beater, French press, or sonicator can be used to burst open these samples. With most the biological samples such as tissue cultures, blood, and buccal cells, an alkaline or chaotropic salt treatment of the sample can be performed to lyse open the cells. The chaotropic salt can be a guanidinium salt, for example, guanidine hydrochloride, guanidine thiocyanate, guanidinium chloride, guanidinium hydrochloride, or guanidinium isothiocyanate.

Other methods for lysis include; enzymatic procedures, boiling methods, organic solvent, and detergent treatments. Efficient lysis increases the detection of low copy number nucleic acids.

To achieve efficient lysis of pathogens and microorganisms, a very robust protocol has been discovered to lyse the cells, denature the genomic DNA, degrade the proteins, and emulsify lipids in a complex sample media. The method includes two enzymatic

treatments: a reagent, such as a lysozyme, to cleave the peptidoglycan membranes, and a protease to degrade the proteins, especially nucleases, which can destroy the nucleic acids in the mixture.

In a preferred embodiment, the reagent used to cleave peptidoglycan bonds is selected from lysozyme, lysostaphin, and mutanolysin. In a particularly preferred embodiment, the reagent is 5 mgs/ml of lysozyme.

In a preferred embodiment, the protease is selected from proteinase K, pronase E, achromopeptidase, proteinase R, proteinase T, subtilisin DY, an alkaline serine protease from *Streptomyces griseus* or *Bacillus lichenformis*, dispase, subtilisin Calsberg, subtilopeptidase A, and thermolysin. In a particularly preferred embodiment, the protease is 1.5 mgs/ml of proteinase K.

In addition, two other treatments can be added to efficiently lyse a low level of pathogens: a detergent treatment to emulsify the lipids and denature the proteins and an alkaline treatment to burst open the cells in the sample and denature the genomic DNA.

Treatment with a NaCl solution may also be included in the lysis protocol. In certain embodiments, the NaCl solution may be of a concentration greater than 250 mM. In preferred embodiments, the NaCl solution may have a concentration of about 500mM to 1M, such as 500mM, 750mM or 1.0M. In other embodiments, the NaCl solution may be of a concentration greater than 1M.

In one embodiment, the detergent is a non-ionic detergent, for example, t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), or sorbitan monolaurate (Span 20). In a preferred embodiment, the nonionic detergent is polyoxyethylenesorbitan monolaurate (Tween-20) at 0.5%-2.0% v/v.

In another embodiment, the detergent is a cationic surfactant, for example, cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), tetradecetyltrimethylammonium bromide (TTAB), tetradecetyltrimethylammonium chloride (TTACl), dodecyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DTACl), dodecylethyldimethylammonium chloride (DEDTAB), decyltrimethylammonium bromide (D10TAB), or dodecyltripheylphosphonium bromide (DTPB).

In a preferred embodiment, the alkaline solution is 50mM-500mM KOH or NaOH. Performing lysis using a lysozyme/protease/alkaline solution protocol, a lysozyme/protease/detergent/alkaline solution protocol, or a lysozyme/protease/NaCl solution protocol in the presence of a metal oxide solid support creates a highly sensitive and consistent nucleic acid sample preparation procedure that can be used for further molecular biology assays. Alternatively, the protocol can be performed without the protease treatment.

METAL OXIDES

Nucleic acids can be bound to metal oxides for purification and detection, particularly by amplification of the nucleic acid bound to the metal oxide. The invention encompasses the use of metal oxides that have the property of being highly electropositive due to hydroxyl groups or other hydrophilic moieties. As used herein, the term "metal oxide" specifically excludes oxides of silicon.

Preferred oxides are oxides of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum, chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, iron, and combinations of these metal oxides. Particularly preferred metal oxides in higher to lower order of affinity of binding/ amplification/ detection of the nucleic acid directly on the beads are aluminum, niobium, iron, zirconium, titanium, antimony, cerium, and germanium.

Metal oxides can be either magnetic or non-magnetic. Metal oxides are preferably 50% pure, more preferably 75%, 85%, 90%, 95%, or 99% pure.

In a preferred embodiment of the invention, the metal oxides are metal oxide beads. In one embodiment, the beads are between 1 and 200 microns in size. In another embodiment, the beads are between 2 and 150 microns in size, more preferably, between 5 and 100 microns in size. Most preferably, the beads are 5, 10, 25, 50, or 100 microns in size.

The metal oxide beads of the invention have the unexpected property of allowing the detection of an amplified product in the same vessel as a DNA is bound and amplified. This property allows for DNA purification, amplification, and detection in the same tube.

SOLID SUPPORT

The metal oxide can be attached to a solid support, such as a plate, column, filter, beads, coating, or a rod. In a preferred embodiment, the metal oxide is attached to a

vessel. As referred to herein, a vessel is a solid support with walls that can contain a solution in an amplification reaction. For example, the vessel can be a well or tube, most preferably a PCR tube. Preferably, the vessel is a plastic vessel. The vessel can include a top or lid.

In one embodiment, the support is coated with a layer of metal oxide, preferably aluminum oxide, at a 100-200 mesh. In another embodiment, the support is coated with metal oxide beads, preferably aluminum oxide beads.

To permit detection of amplification products in the same vessel, the concentration of beads must be sufficiently high to bind a detectable quantity of DNA and sufficiently low so as not to interfere with amplification and/or detection. Using higher concentrations of beads can lead to increased binding of primers or probes. Using higher concentrations of beads can also interfere with the ability to detect the signal in the same tube. The precise limits at which detection is no longer possible varies with the size and concentration of the beads in the tube, as well as the level of nucleic acid to be detected, and can be determined empirically, for example, by using the procedures set forth in the examples. As a standard for such determinations, the use of 15 mgs. of 100 micron aluminum hydroxide beads in a 250 µl PCR tube allows for detection of amplification products in the same vessel.

In a preferred embodiment, a vessel contains 5-50 mgs. of metal oxide beads in an area in contact with a 50 µl reaction volume. More preferably, the vessel contains 10-25 mgs. of metal oxide beads. Most preferably, the vessel contains 15 mgs. of metal oxide beads.

A vessel coated with a metal oxide includes a vessel having the metal oxide embedded in the vessel walls. Attachment can be performed using any method that attaches the metal oxide to the vessel and allows DNA binding to the metal oxide, while not interfering with amplification or detection. For example, attachment can be achieved using an adhesive, by chemical or vapor deposition, by propelling the metal oxide beads into a plastic vessel at high velocity, or by heating the metal oxide beads prior to contacting them with a plastic vessel. In one embodiment, metal oxide beads are heated to 800° C prior to addition to a plastic vessel. In this manner, the beads are imbedded into the plastic vessel. Examples of methods for binding metal oxide beads to vessels can be found in WO/03020981, the specific methods of which are hereby incorporated by reference.

In a particularly preferred embodiment, the metal oxide is coated onto a vessel that allows for binding of nucleic acid to the metal oxide, amplification of the bound nucleic

acid, and detection of the amplified products in the same vessel. Detection of the amplified products can be real-time (during the amplification process) or endpoint (after the amplification process). The invention allows the detection of amplification in the same vessel that is used for purification of nucleic acid. Detection of amplification in the presence of the metal oxide is a novel and unexpected result of the invention.

NUCLEIC ACID BINDING TO METAL OXIDE SUPPORT

The sample conditions for binding nucleic acids to the metal oxide support encompass a wide range of aqueous conditions. This includes a broad range of buffered solution, broths, pH, and detergents. Preferred binding conditions are dictated by the lysis conditions that aid in promoting high affinity binding of nucleic acids to the metal oxide. Preferably, binding of the nucleic acids is performed under alkaline conditions with a detergent. Binding of the nucleic acids may also be performed in NaCl concentrations of greater than 250mM. Preferentially, the binding/lysis buffer ranges in pH from 7-12, more preferably, 8-12. The binding buffer can contain a detergent, for example, non-ionic detergents, ionic detergents, and zwitterionic detergents, at a concentration of 0.1-25%. Preferably, 0.5-2% of Tween-20 is used in the binding/ lysis buffer. After the binding/lysis buffer is added to the sample, the nucleic acid can be agitated over the metal oxide support by rotating, rocking, shaking, vortexing, or pipetting up and down to allow sufficient contact of the substrate with the solid support. Once the sample has been mixed/ agitated thoroughly over the support, the sample can be removed and discarded.

WASHING OF BOUND NUCLEIC ACIDS

The solid support bound with the nucleic acid can be subjected to a wash buffer to remove residual inhibitors from the sample, without dissociating the bound nucleic acid. Solutions for washing the solid support include water, buffered detergents, alcohols, and chelexing agents. The type and concentration of detergents can be similar to those described above. Preferred alcohols are methanol, ethanol, and isopropanol at a 20-70% concentration.

In one embodiment, the wash buffer includes a detergent, such as Tween-20, in a Tris pH8.0 buffer. The wash can be performed by adding the wash solution to the metal oxide then mixing/agitating the solution by pipetting, vortexing, rotating, or rocking. The

wash can be performed one, two, three, or more, times. In a preferred embodiment, two washes are performed.

In one embodiment, the wash is performed with a buffer that comprises t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), sorbitan monolaurate (Span 20), methanol, ethanol, or isopropanol. In a preferred embodiment, the buffer comprises at least 5% w/w ethanol and at least 0.5% w/w polyoxyethylenesorbitan monolaurate (Tween-20).

ELUTION OF NUCLEIC ACIDS FROM SUPPORT

Elution of the nucleic acid from the support is not necessary for the detection of pathogens. Rather, molecular biological reactions, such as PCR, which amplify the bound nucleic acids, can be performed on the solid support directly. However, if desired, the nucleic acids can be eluted from the support with elution buffers. Elution buffers can consist of Tris/EDTA pH 8.0, deionized water, or a phosphate buffer. For high concentration of target substrate, elution will not interfere with downstream reactions, but for low concentration such as 1-100 copies of target sequence, elution may not be able to extract enough material for downstream processing. In a particularly preferred embodiment, no elution of the nucleic acid is performed.

DETECTION OF NUCLEIC ACIDS ON THE SUPPORT

Nucleic acids bound to a support can be detected directly or can be detected during or after an amplification reaction.

Direct detection

When high copy numbers of specific nucleic acids are present in the sample, high copy numbers of the nucleic acids can be bound to the support. A high copy number of a specific nucleic acid can allow its detection directly on the support without any amplification of the nucleic acid. The nucleic acid can be directly detected with standard hybridization-based technologies, for example, using a radiolabeled, fluorescently labeled, chemiluminescently labeled, or enzymatically labeled probe.

Nucleic Acid Amplification

With low copy numbers of pathogens, amplification of the specific target sequence can be performed to increase the number of copies of nucleic acid for detection. "Amplification," as referred to herein, means the generation of copies of a nucleic acid. A copy may comprise DNA, RNA, or a modified nucleic acid. Copies generated by an amplification method are referred to herein as "amplified product."

Nucleic acids bound to metal oxides can be amplified with many varied amplification methods and amplification reaction conditions. For example, the amplification method can be rolling circle amplification (RCA), self-sustained sequence replication (3SR), multiple displacement amplification MDA, Nucleic acid sequence based amplification (NASBA), Transcription-mediated amplification (TMA), strand displacement amplification (SDA), Ligase chain reaction (LCR), b-DNA amplification, polymerase chain reaction (PCR, all forms including RT-PCR), ramification amplification RAM, loop-mediated isothermic amplification LAMP, isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN), Isothermal Single Primer Amplification (SPIA), QB-replicase mediated amplification, or Invader assay.

A preferred amplification method is the polymerase chain reaction (PCR) amplification. *See, e.g.*, *PCR Technology: Principles and Applications for DNA Amplification* (Ed. H. A. Erlich, Freeman Press, NY, N.Y., 1992); *PCR Protocols: A Guide to Methods and Applications* (Eds. Iinis, et al., Academic Press, San Diego, Calif., 1990); Mattila et al., *Nucleic Acids Res.* 19, 4967 (1991); Eckert et al., *PCR Methods and Applications* 1, 17 (1991); *PCR* (Eds. McPherson et al., IRL Press, Oxford); and U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159 4,965,188, and 5,333,675.

In a particularly preferred embodiment, the amplification reaction is a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min.

Other preferred amplification methods include the ligase chain reaction (LCR) (e.g., Wu and Wallace, *Genomics* 4, 560 (1989), Landegren et al., *Science* 241, 1077 (1988) and Barringer et al. *Gene* 89:117 (1990)), transcription amplification (Kwoh et al., *Proc. Natl. Acad. Sci. USA* 86, 1173 (1989) and WO88/10315), self-sustained sequence replication (Guatelli et al., *Proc. Nat. Acad. Sci. USA*, 87, 1874 (1990) and WO90/06995), selective amplification of target polynucleotide sequences (U.S. Pat. No. 6,410,276), and nucleic

acid sequence based amplification (NASBA) (U.S. Pat. Nos. 5,130,238, 5,409,818, 5,554,517, and 6,063,603). Other amplification methods that may be used are described in U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and 6,582,938. The above references regarding amplification of nucleic acids are specifically incorporated by reference with respect to the disclosure therein of the specific reaction conditions used for amplification in each of the amplification methods.

Detection of amplified product

In one embodiment, amplified product is detected by detecting the generation of double-stranded nucleic acid, such as with a fluorescent intercalating dye, e.g., SYBR green. In this embodiment, the signal generated by the intercalating dye corresponds to the level of amplified product. Thus, detection of a signal can represent the attachment of a specific nucleic acid to the support. In a particularly preferred embodiment, detection is performed during amplification. In another preferred embodiment, detection is performed after amplification.

In another embodiment, the amplified product is detected with a specific detection chemistry such as fluorescence resonance energy transfer (FRET) probes, Taqman probes, Eclipse MGB probes, molecular beacons, scorpion probes, fluorescently labeled primers, lightup probes or a dye-based chemistry, DNA, PNA, LNA, RNA, or o-methyl RNA, including modified bases that bind to the amplified product to detect the sequence of interest.

Detection of the amplified products can be real-time (during the amplification process) or endpoint (after the amplification process). The invention allows for detection of the amplification products in the same vessel in which the nucleic acid is bound to the metal oxide.

An enhancing reagent can be used to enhance amplification reactions by preventing the binding of primers, oligomers, or other amplification reagents to the solid support. This enhancing reagent contains a viscous component and a phosphate at a concentration sufficient to block the remaining solid support from binding nucleic acid reagents in the amplification reactions. Preferably, the viscous component comprises glycerol at 1-20% v/v. Preferably, the phosphate concentration is 20-200mM.

The amplified nucleic acid can also be used for other molecular biology assays, including but not limited to, sequencing, cloning, restriction enzyme digest, RFLP, and any other manipulations of the nucleic acids.

COMPOSITIONS

The invention includes compositions for purification and detection of nucleic acids. These compositions are useful in the above-described methods.

For example, the invention includes reagents for the preparation of nucleic acids from cells as described above. The invention also includes reagents for the purification of nucleic acids, as described above. Such reagents include vessels, such as PCR tubes and wells coated with metal oxides.

In one embodiment, the composition comprises a 250µl PCR tube coated with aluminum oxide beads. In a preferred embodiment, the beads are between 5 and 100 microns in size. In a particularly preferred embodiment, the tubes are coated with 15 mgs. of 5 micron beads. In one embodiment, the beads are imbedded into the walls of the PCR tube by heating them to 800°C and contacting them with the tube. The coated tubes of the invention have the capacity to be used for DNA purification, amplification, and detection of amplified products in the same tube.

KITS

The invention encompasses kits for the preparation, amplification, and/or detection of nucleic acids. The kit can include sample preparation reagents, amplification reagents, and/or detection reagents. The kit can include a reagent for lysis of a sample, which can be either in a solution or lyophilized form. The lysis reagent can contain lysozyme, protease, a detergent, a high alkaline solution and/or a NaCl solution. The lysis reagent may be comprised of multiple components that are added to the sample at the same time or consecutively.

In a preferred embodiment, the kit contains nucleic acid capture and concentration vessel, for example, a PCR tube or a well, that has an aluminum oxide coating on the interior surface. The kit can include an enhancing reagent. The kit can include a wash solution and reagents for an amplification reaction. For example, the kit can contain PCR primers and probes specific for microorganisms of interest. Microorganisms of interest include *E. coli* (*e.g., E. coli* O157:H7), *Listeria (e.g., L. monocytogenes), Salmonella, Campylobacter, Legionella, (e.g., L. pneumophila) Bacillus (e.g., B. anthracis), Mycobacterium (e.g., M. tuberculosis), Mycoplasma, Clostridium, Chlamydia, Proteus, Enterococcus, Enterobacter, Pseudomonas, Neisseria, Staphylococcus (e.g., S. aureus), Streptococcus, Vibrio, Shigella, Helicobacter (e.g., H. pylori), Clostridium, Yersinia, Haemophilus, Pneumococcus,* and *Seratia*. Other microorganisms of interest

include viruses, for example, human immunodeficiency viruses (*e.g.,* HIV-1), hepatitis viruses (*e.g.*, Hepatitis B virus), herpesviruses, rhinoviruses, papillomaviruses, and adenoviruses. In a preferred embodiment, the microorganism is a human pathogen.

The kit can include all, or some, reagents to lyse, purify, concentrate, amplify, and detect a specific nucleic acid in a complex biological sample. In a preferred embodiment, the specific nucleic acid is at a concentration of between 1 and 1000 copies/ml, preferably between 10 and 100 copies/ml. In one embodiment, the concentration of the specific nucleic acid is at least 100 copies /ml.

In a preferred embodiment, the kit will be able to purify, amplify, and detect nucleic acids in a single vessel. The kit can contain instructions for purifying, amplifying, and detecting nucleic acids in a single vessel.

The invention is more fully understood with reference to the following examples.

Example 1

DNA binding to metal oxides

DNA binding to different metal oxides was measured by placing 15 mgs. of less than 100micron metal oxide particles in the presence of 10 or 1 million copies of *Listeria monocytogenes* genomic DNA. The DNA was diluted in 100µl of 0.5% Tween-20 solution to the specific concentration. This solution enhances binding of nucleic acids to the metal oxides. The DNA/metal oxide slurry was rotated at room temperature for 10 mins. The particles were spun down to pellet the metal oxides and 34µls of the solution were added to a real-time PCR reaction to quantitate the concentration of DNA that remained in solution unbound to the particles. The sequence of the primers and probes used in this experiment are shown below:

Sequence 1: TCGTGCGCTTCTAGGT (SEQ ID NO:1).

Sequence 2: TGCTTTAGTTGCGATGGA (SEQ ID NO:2).

Probe Sequence: TATGAGTCGCCTTAGCTACAATGTATCT (SEQ ID NO:3).

Target Sequence:

AATTACTAGATCAAACTGCTACAGGTGCTGCTACTCAAGTAAGCATCCAAGCGTCT
GATAAAGCTAATGACTTAATCAATATCGATCTTTTCAACGCTAAAGGTCTTTCTGCT
GGAACAATCACTTTAGGTAGTGGTTCTACAGTTGCTGGTTATAGTGCATTATCCGTT
GCTGATGCTGATTCTTCTCAACAAGCAACTGAAGCTATTGATGAATTAATCAATAAC
ATTTCTAACGGTCGTGCGCTTCTAGGTGCTGGTATGAGTCGCCTTAGCTACAATGT
ATCTAACGTGAACAATCAATCCATCGCAACTAAAGCATCTGCTTCATCCATTGAAGA

TGCAGATATGGCTGCTGAAATGTCCGAAATGACTAAATACAAAATTCTTACACAAAC TTCTATCAGCATGCTTTCTCAAGCAAACCAAACACCGCAAATGTTAACTCAATTAAT TAACAGCTAA (SEQ ID NO:4).

The Taqman™ real-time PCR reaction contained 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 1.5 units of AmpliGold™ Taq using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min.

The percent of DNA bound to the metal oxides was determined by subtracting the concentration unbound from the total DNA concentration and then dividing by the total DNA concentration. Table 2 compares the binding affinities of different metal oxides. Iron, Aluminum, Zirconium, Antimony, and Cerium oxides bound >95% of *Listeria monocytogenes* gDNA at ten and one million copies / 100µls.

Table 2

Metal Oxide	Size of particles	DNA	% Capture	Metal Oxide	Size of particles	DNA	% Capture
Al	100-200	1mil	93.5	Nb	5micron	10m	56.4325
			92.4				59.3737
		100K	99.6			1mil	39.7113
			100.0				31.2332
Al	5micron	10mi	100.0	Sb	5micron	10m	99.9999
			100.0				99.9999
		1mil	100.0			1mil	100
			100.0				99.9998
Zr	5micron	10mi	100.0	Ge	5micron	10m	33.0193
			100.0				5.07552
		1mil	99.9			1mil	64.1473
			100.0				62.2098
Sn	5micron	10mi	37.7	Ce	5micron	10m	99.9804
			59.4				99.8715
		1mil	73.7			1mil	99.9069
			75.8				99.9485
Ti(III)	5microns	1 mil	76.1	Fe(III)	~5micron	1	100.0
			64.4				100.0
		100K	78.5			100	100.0
			39.8				100.0

Example 2

Amplification of DNA bound to metal oxides

Amplification of the DNA bound to the metal oxide was examined using an ethidium bromide stained agarose gel. The DNA was bound to different metal oxides Aluminum (Sigma-Aldrich), Titanium (EM), Iron (SK Magnetics), Zirconium, Niobium, Germanium, Antimony, and Cerium (Alfa Aesar) in the presence of 40mM NaOH and 0.5% Tween-20 solution. The alkaline solution was used to denature the DNA before binding to the metal oxides.

The DNA was titrated from 1 million copies/100µls to 10 copies/100µls in the presence of 15mgs of particles. The DNA/metal oxide slurry was rotated at room temperature for 10 mins. The particles were spun down to pellet the metal oxides and the supernatant was discarded. The metal oxides were washed twice with a 0.5% Tween-20 solution and pelleted to discard the wash solution.

A 50µl PCR reaction was add to the metal oxides bound with different concentrations of the genomic DNA. The reaction contained 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, and 1.5 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min.

A glycerol/ gel loading buffer was added to each PCR reaction and 18µls of the PCR reaction was loaded onto a 1.2% agarose gel. An image of the agarose gel is seen in Figure 1. Certain metal oxides allowed maximal amplification from the DNA bound to the solid support: Aluminum, Titanium, Iron, Zirconium, Niobium, Germanium, Antimony, and Cerium. Thus, amplification from the DNA bound to a solid support can occur efficiently.

Example 3

Real time detection of DNA binding to aluminum oxide coated PCR tubes

75-100µls of a guanidine salt solution were incubated in a PCR tube coated with 15mgs of 100-200um aluminum oxide beads for 5 mins at room temperature to render the metal oxides hydrophilic. The genomic DNA was added after this treatment at different concentrations to determine the amount of DNA that is bound to the tubes. The sample was pipetted up and down 10 times and then discarded. The tube was washed and then PCR reagents were added on top of the solid matrix (Fig. 2).

Alternatively, genomic DNA was added straight to the tubes without rendering it hydrophilic. The sample was pipetted up and down 10 times and then discarded. The tube was washed two times with 200µls of wash buffer (Fig. 2).

Each protocol was analyzed by a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, Rox reference dye and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min. Results are shown as a plot of cycle time vs. FAM signal.

The pretreatment of aluminum oxide is not necessary for DNA binding. The metal oxide does not need to be rendered hydrophilic before binding genomic DNA. Detection can occur in the same tube as the DNA binding and amplification reactions.

Example 4

Improving lysis of bacteria can increase DNA binding + to aluminum oxide coated PCR tubes

75-100µls of a guanidine salt solution were incubated in a PCR tube coated with 15mgs of 100-200um aluminum oxide beads for 5 mins at room temperature. A food sample post-spiked with a predetermine concentration of *Listeria monocytogenes* was added to the tube, mixed by pipetting 5 times, and allowed to incubate at room temperature for 10 mins. The lysed sample was mixed 10 times over the aluminum oxide to capture the genomic DNA. The original sample was discarded and the aluminum oxide was washed two times with 200µls of wash buffer (Fig. 3).

For comparison, 180µls of a food sample post-spiked with a predetermined concentration of *Listeria monocytogenes* were added to the tubes and 5mgs/ml of lysozyme. The sample was incubated 10 mins at room temperature. Next, 0.5% Tween-20 and 1.5mgs/ml of proteinase K were added to the sample and was incubated for 20mins at 55°C. 200mM of NaOH was added and the sample was immediately pipetted 10 times, and then discarded. The tube was washed 2 times with a Tween-20 solution (Fig. 4).

Alternatively, depending on the sample type and the amount of protein, 180µls of sample were treated with only 5mg/ml lysozyme for 10 mins at room temperature and no protease treatment was employed. The effect of the protease treatment was dependent on the fat content of the ground beef sample. 200mM of NaOH and 0.5% Tween-20 was added to the sample and mixed by pipetting ten times in a PCR tube

with aluminum oxide coating. The sample was discarded and the aluminum oxide bound with genomic DNA was washed 2 times with 120µls of a Tween-20 wash solution (Fig. 5).

Each protocol was analyzed by a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min. Results are shown as a plot of cycle time vs. FAM signal.

The lysozyme/protease/detergent/alkaline solution and lysozyme/detergent/alkaline solution protocols improved the ability to capture, concentrate and detect genomic DNA from gram(+) bacteria at low concentration.

Example 5

Real time detection of *E. coli* O157:H7 DNA binding to aluminum oxide coated PCR tubes

The same protocols were performed as in Example 3 using *E. coli* O157:H7. Figure 7 is the improved lysis protocol with lysozyme/protease/detergent/alkaline solution. Each protocol was analyzed by a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min. Results are shown as a plot of cycle time vs. FAM signal. The lysis protocol with lysozyme/protease/detergent/alkaline solution improved the ability to capture, concentrate and detect genomic DNA from gram(-) bacteria at low concentration.

Example 6

Lysozyme treatment can improve DNA binding to aluminum oxide coated PCR tubes

Lysozyme treatment can improve DNA binding to metal oxides. A protocol with and without lysozyme was performed to determine the effect of this enzyme. A 180µl food sample, post-spiked with a predetermined concentration of *Listeria monocytogenes,* and 5mgs/ml of lysozyme were added to tubes coated with 15mgs of 100-200um aluminum oxide beads. The sample was incubated 10 mins at room temperature. Next, 0.5% Tween-20 and 1.5mgs/ml of proteinase K were added to the sample and was incubated

for 20 mins at 55°C. 200mM of NaOH was added and the sample was immediately pipetted 10 times, and then discarded. The tube was washed 2 times with a Tween-20 solution (Figure 8).

Alternatively, 180µls of sample were added to PCR tubes coated with aluminum oxide and treated with protease and 0.5% Tween-20 solution for 20 mins at 55°C. 200mM of NaOH was added to the sample and mix by pipetting ten times in a PCR tube with aluminum oxide coating. The sample was discarded and the aluminum oxide bound with genomic DNA was washed 2 times with 120µls of a Tween-20 wash solution (Figure 8).

Each protocol was analyzed by a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min. Results are shown as a plot of cycle time vs. FAM signal.

The ability to consistently extract, capture, and detect genomic DNA from bacteria was improved with lysozyme.

Example 7

Proteinase K treatment can improve DNA binding to aluminum oxide coated PCR tubes

Protease treatment can improve DNA binding to metal oxides. A protocol with and without protease was performed to determine the effect of this enzyme. A 180µl food sample, post-spiked with a predetermined concentration of *Listeria monocytogenes*, and 5mgs/ml of lysozyme were added to the tubes coated with 15mgs of 100-200um aluminum oxide beads. The sample was incubated 10 mins at room temperature. Next, 0.5% Tween-20 and 1.5mgs/ml of proteinase K were added to the sample and the sample was incubated for 20 mins at 55°C. 200mM of NaOH was added and the sample was immediately pipetted 10 times, and then. The tube was washed 2 times with a Tween-20 solution (Figure 9).

Alternatively, 180µls of sample were added to PCR tubes coated with aluminum oxide and treated with 5mgs/ml of lysozyme for 10 mins at room temperature. A final concentration of 200mM of NaOH and 0.5% Tween-20 was added to the sample and mix by pipetting ten times in a PCR tube with aluminum oxide coating. The sample was

discarded and the aluminum oxide bound with genomic DNA was washed 2 times with 120µls of a Tween-20 wash solution (Figure 9).

Each protocol was analyzed by a Taqman™ real-time PCR reaction containing 1.5mM $MgCl_2$, 200µM dNTPs, 200nM each primer pair, 200nM Taqman™ probe, and 2 units of AmpliGold™ Taq with reagents using a thermocyler profile of 95°C for 10 mins, and fifty cycles of 95°C 15 secs and 55°C for 1 min. Results are shown as a plot of cycle time vs. FAM signal.

Example 8

Primer concentrations can improve detection of DNA bound to aluminum oxide coated PCR tubes

PCR primer and probes can be added at a high concentration in order to increase the ability to consistently lyse and detect micro-organisms in a single vessel. Micro-organisms were lysed by adding 180µls of a food sample post-spiked with a predetermine concentration of *Listeria monocytogenes* and 5mgs/ml of lysozyme to the tubes coated with aluminum oxide. The sample was incubated 10 mins at room temperature. Next, 0.5% Tween-20 and 1.5mgs/ml of proteinase K were added to the sample and is incubated for 20 mins at 55°C. 200mM of NaOH were added and the sample was immediately pipetted 10 times, and then discarded. The tubes were washed 2 times with a Tween-20 solution.

Four different Taqman™ PCR mastermixes were made to determine the optimal concentrations of primers to improve the amplification reaction. Figure 10 shows the concentration of 200nM of both primers and probes. Figure 11 shows the results of a PCR reaction with 800nM of primers and 200nM of probes. Figure 12 shows the results of a PCR mix with 800nM of the forward Primer and 200nM of the reverse primer and probe. Lastly, Figure 13 displays the results of the PCR mix with 800nM of the reverse primer and 200nM of the forward primer and probe. Adding high concentrations of either forward or reverse primers increased the accuracy of amplification and detection of the specific genomic DNA bound to the solid matrix.

Example 9

Sodium chloride can be used in place of sodium hydroxide for detection of DNA bound to aluminum oxide coated PCR tubes

Sodium chloride (NaCl) is an alternative to sodium hydroxide (NaOH) for bacterial lysis and binding of released DNA onto aluminum oxide. Results were compared for samples tested with protocols using either NaCl or NaOH. Twelve 180µl environmental sponge samples, post-spiked with approximately 180 cells of *Listeria monocytogenes*, were added to tubes coated with 15mg of 100-200µm aluminum oxide beads. Four negative control samples that were the same environmental sample without post-spike were also added to coated tubes. To each tube, 1mg of lysozyme and 20µg of pronase E were added, the tubes were sealed with plastic film and the tubes were incubated for 30 minutes at 60°C. Next, 20µL of 4M NaOH was added to six of the spiked tubes and two of the negative controls tubes and 20µL of 5M NaCl was added to the other six spiked tubes and the other two negative control tubes. The contents of each tube was immediately pipetted up and down 10 times, and then removed and discarded. The tubes were then washed two times with 150µL of distilled water.

Each sample was analyzed by a real-time PCR reaction using a commercial multiplex PCR reagent kit (QuantiTect® Multiplex, Qiagen, Inc. Valencia, CA). Each reaction contained 25µL of 2X PCR mix from the kit along with 2.5µL primer mix, 2.5µL of fluorescent probe, and 1.3µL (1000 copies) of an internal control template in a final volume of 50µL. The thermal cycling profile consisted of a hold at 50°C for 2 minutes, a hold at 95°C for 15 minutes, a hold at 56°C for 30 seconds, then fifty cycles of 95°C for 5 seconds, 56°C for 30 seconds, and 76°C for 20 seconds.

Figure 14 shows a plot of cycle time vs. FAM signal for the NaOH and NaCl samples. The appearance of fluorescent product occurred at cycle 34 to 37 for all spiked samples while the negative control samples show either no distinct fluorescence increase or show increases after cycle 41. The results indicate that NaCl can be substituted for NaOH in the present invention without significant impact on DNA capture and amplification.

Example 10

Sodium chloride concentration optimization for detection of DNA bound to aluminum oxide coated PCR tubes

Samples containing a range of sodium chloride (NaCl) concentrations were tested to determine the concentration range for optimal bacterial lysis and binding of released DNA onto aluminum oxide. Results were compared for samples tested with protocols using 0.05 to 1M NaCl. Five 180µl environmental sponge samples, post-spiked with

approximately 18 cells of *Listeria monocytogenes*, were added to tubes coated with aluminum oxide beads. To each tube, 1mg of lysozyme and 20µg of pronase E were added, the tubes were sealed with plastic film and the tubes were incubated for 30 minutes at 60°C. Next, NaCl was added to one tube each to a final concentration of 0.05, 0.1, 0.25, 0.5, and 1.0M, respectively, and the tube contents were immediately pipetted up and down 10 times, and then removed and discarded. The tubes were then washed two times with 150µL of distilled water.

Each sample was analyzed by a real-time PCR reaction using a commercial multiplex PCR reagent kit (QuantiTect® Multiplex, Qiagen, Inc. Valencia, CA). Each reaction contained 25µL of 2X PCR mix from the kit along with 2.5µL primer mix, 2.5µL of fluorescent probe, and 1.3µL (1000 copies) of an internal control template in a final volume of 50µL. The thermal cycling profile consisted of a hold at 50°C for 2 minutes, a hold at 95°C for 15 minutes, a hold at 56°C for 30 seconds, then fifty cycles of 95°C for 5 seconds, 56°C for 30 seconds, and 76°C for 20 seconds. Figure 15 shows a plot of cycle time vs. FAM signal. The appearance of fluorescent product occurred at cycle 35 for the 0.5M NaCl sample and at cycle 33 for the 1.0M NaCl sample.

The results indicate that NaCl is effective in the process of the present invention at concentrations of 0.5M and greater. Subsequent testing indicated that 0.75M NaCl was also effective.

It will be apparent to the skilled artisan that the above description is merely exemplary and that the invention encompasses various modifications known to the skilled artisan.

We claim:

1. A method for the detection of a microorganism comprising:

(a) lysing a microorganism to release a nucleic acid molecule;

(b) contacting the nucleic acid molecule with a vessel coated with at least one metal oxide;

(c) allowing the nucleic acid molecule to attach to the metal oxide;

(d) amplifying, in the vessel, a fragment of the nucleic acid molecule attached to the metal oxide to generate an amplified product; and

(e) detecting, in the vessel, the amplified product.

2. The method of claim 1, wherein the microorganism is lysed in step (a) with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, and an alkaline solution.

3. The method of claim 1, wherein the microorganism is lysed in step (a) with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, a detergent, and an alkaline solution.

4. The method of claim 1, wherein the microorganism is lysed in step (a) with a reagent that cleaves the peptidoglycan bonds on the outer membrane, a protease, and a NaCl solution of a concentration greater than 250mM.

5. The method of claim 2, wherein the reagent that cleaves peptidoglycan bonds is lysozyme, lysostaphin, or mutanolysin.

6. The method of claim 5, wherein the reagent that cleaves peptidoglycan bonds is lysozyme.

7. The method of claim 2, wherein the protease is proteinase K, pronase E, achromopeptidase, proteinase R, proteinase T, subtilisin DY, an alkaline serine protease from *Streptomyces griseus* or *Bacillus lichenformis*, dispase, subtilisin Calsberg, subtilopeptidase A, or thermolysin.

8. The method of claim 7, wherein the protease is proteinase K.

9. The method of claim 2, wherein the alkaline solution is 50mM-500mM KOH or NaOH.

10. The method of claim 3, wherein the detergent is t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), or sorbitan monolaurate (Span 20).

11. The method of claim 10, wherein the detergent is polyoxyethylenesorbitan monolaurate (Tween-20).

12. The method of claim 1, wherein the microorganism is lysed in step (a) with a chaotropic salt or a cationic surfactant.

13. The method of claim 12, wherein the chaotropic agent is a guanidinium salt.

14. The method of claim 13, wherein the guanidinium salt is guanidine hydrochloride, guanidine thiocyanate, guanidinium chloride, guanidinium hydrochloride, or guanidinium isothiocyanate.

15. The method of claim 12, wherein the cationic surfactant is cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), tetradecetyltrimethylammonium bromide (TTAB), tetradecetyltrimethylammonium chloride (TTACl), dodecyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DTACl), dodecylethyldimethylammonium chloride (DEDTAB), decyltrimethylammonium bromide (D10TAB), or dodecyltripheylphosphonium bromide (DTPB).

16. The method of claim 1, wherein the metal oxide is an oxide of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum,

chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, or iron.

17. The method of claim 16, wherein the metal oxide is aluminum oxide.

18. The method of claim 1, wherein the vessel is a tube, column, plate, or well.

19. The method of claim 18, wherein the vessel is a PCR tube.

20. The method of claim 1, where the attached nucleic acid molecule is washed with a buffer that comprises t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylenesorbitan monolaurate (Tween-20), polyoxyethylenesorbitan monopalmitate (Tween-40), polyoxyethylenesorbitan monostearate (Tween-60), polyoxyethylenesorbitan monooleate (Tween-80), polyoxyethylenesorbitan monotrioleate (Tween-85), (octylphenoxy)polyethoxyethanol (IGEPAL CA-630), triethyleneglycol monolauryl ether (Brij 30), sorbitan monolaurate (Span 20), methanol, ethanol, or isopropanol.

21. The method of claim 20, wherein the buffer comprises at least 5% w/w ethanol and at least 0.5% w/w polyoxyethylenesorbitan monolaurate (Tween-20).

22. The method of claim 1, wherein the amplified product is detected by hybridization to a probe.

23. The method of claim 22, wherein the amplified product is detected by hybridization to a labeled DNA, PNA, RNA, o-methyl RNA, LNA, or modified nucleotide probe.

24. The method of claim 23, wherein the probe is fluorescently labeled.

25. The method of claim 1, wherein the amplified product is detected with an intercalating dye.

26. The method of claim 1, wherein the nucleic acid molecule is amplified by the polymerase chain reaction (PCR).

27. The method of claim 26, wherein amplification is detected by hybridization using a probe.

28. The method of claim 27, wherein the amplified product is detected by hybridization to a labeled DNA, PNA, RNA, o-methyl RNA, LNA, or modified nucleotide probe.

29. The method of claim 28, wherein the probe is fluorescently labeled.

30. The method of claim 26, wherein amplification is detected with an intercalating dye.

31. The method of claim 26, wherein the amplified product is detected during amplification.

32. The method of claim 26, wherein the nucleic acid molecule is amplified with a fluorescently labeled primer.

33. A kit for detecting nucleic acids in biological sample comprising:

(a) a vessel coated with at least one metal oxide;

(b) instructions for binding a nucleic acid molecule to the metal oxide; and

(c) instructions for amplifying and detecting, in the vessel, a nucleic acid molecule bound to the metal oxide.

34. The kit of claim 33, further comprising primers for amplifying a nucleic acid molecule bound to the metal oxide.

35. The kit of claim 33, further comprising a fluorescent probe.

36. The kit of claim 33, further comprising a lysozyme, a protease, and an alkaline solution.

37. The kit of claim 36, further comprising a nonionic detergent.

38. The kit of claim 33, further comprising a lysozyme, a protease, and a NaCl solution of a concentration greater than 250mM.

39. The kit of claim 33, wherein the metal oxide is an oxide of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum, chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, or iron.

40. The kit of claim 39, wherein the metal oxide is aluminum oxide.

41. The kit of claim 33, wherein the vessel is a tube, column, plate, or well.

42. The kit of claim 40, wherein the vessel is a PCR tube.

43. A kit for detecting nucleic acids in biological sample comprising:
(a) a vessel coated with at least one metal oxide and
(b) instructions for practicing the method of claim 1.

44. The kit of claim 43, further comprising primers for amplifying a nucleic acid molecule bound to the metal oxide.

45. The kit of claim 43, further comprising a fluorescent probe.

46. The kit of claim 43, further comprising a lysozyme, a protease, and an alkaline solution.

47. The kit of claim 46, further comprising a nonionic detergent.

48. The kit of claim 43, further comprising a lysozyme, a protease, and a NaCl solution of a concentration greater than 250mM.

49. The kit of claim 43, wherein the metal oxide is an oxide of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum,

chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, or iron.

50. The kit of claim 49, wherein the metal oxide is aluminum oxide.

51. The kit of claim 43, wherein the vessel is a tube, column, plate, or well.

52. The kit of claim 51, wherein the vessel is a PCR tube.

53. A method for the detection of a nucleic acid comprising:

(a) attaching a nucleic acid molecule to a metal oxide coated onto a vessel;

(b) amplifying, in the vessel, a fragment of the nucleic acid molecule to generate an amplified product; and

(c) detecting, in the vessel, the amplified product.

54. The method of claim 53, wherein the metal oxide is an oxide of aluminum, titanium, zirconium, hafnium, scandium, yttrium, lanthanum, vanadium, tantalum, chromium, molybdenum, tungsten, boron, gallium, indium, germanium, tin, zinc, nickel, magnesium, or iron.

55. The method of claim 53, wherein the metal oxide is aluminum oxide.

56. The method of claim 55, wherein the vessel is a tube, column, plate, or well.

57. The method of claim 56, wherein the vessel is a PCR tube.

58. The method of claim 53, wherein the amplified product is detected by hybridization to a probe.

59. The method of claim 58, wherein the amplified product is detected by hybridization to a labeled DNA, PNA, RNA, o-methyl RNA, LNA, or modified nucleotide probe.

60. The method of claim 59, wherein the probe is fluorescently labeled.

61. The method of claim 53, wherein the amplified product is detected with an intercalating dye.

62. The method of claim 53, wherein the nucleic acid molecule is amplified by the polymerase chain reaction (PCR).

63. The method of claim 62, wherein amplification is detected by hybridization using a probe.

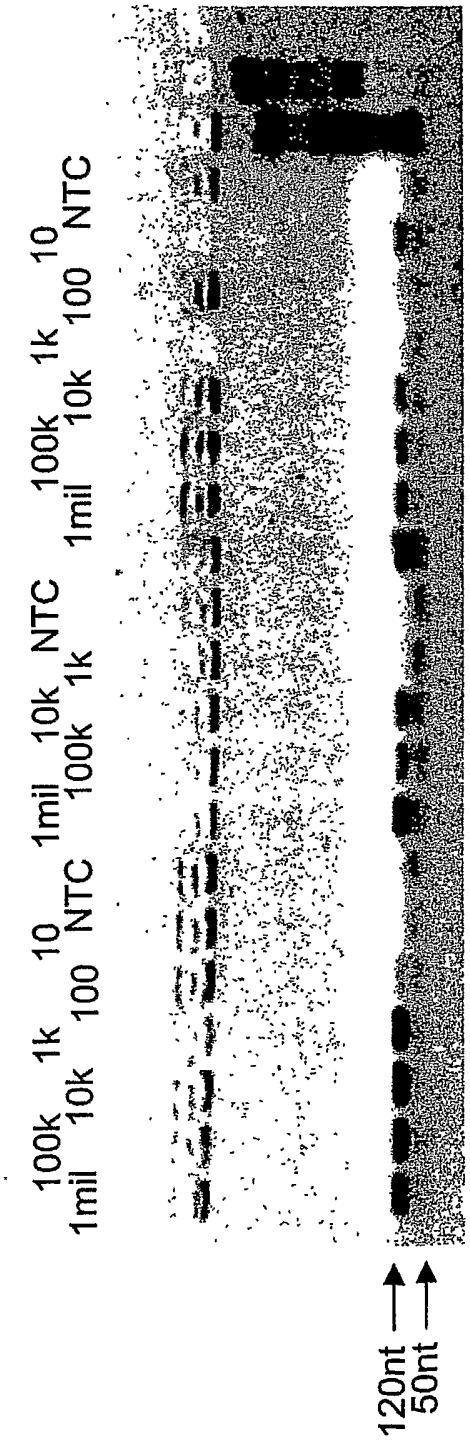
64. The method of claim 63, wherein the amplified product is detected by hybridization to a labeled DNA, PNA, RNA, o-methyl RNA, LNA, or modified nucleotide probe.

65. The method of claim 64, wherein the probe is fluorescently labeled.

66. The method of claim 62, wherein amplification is detected with an intercalating dye.

67. The method of claim 62, wherein the amplified product is detected during amplification.

68. The method of claim 62, wherein the nucleic acid molecule is amplified with a fluorescently labeled primer.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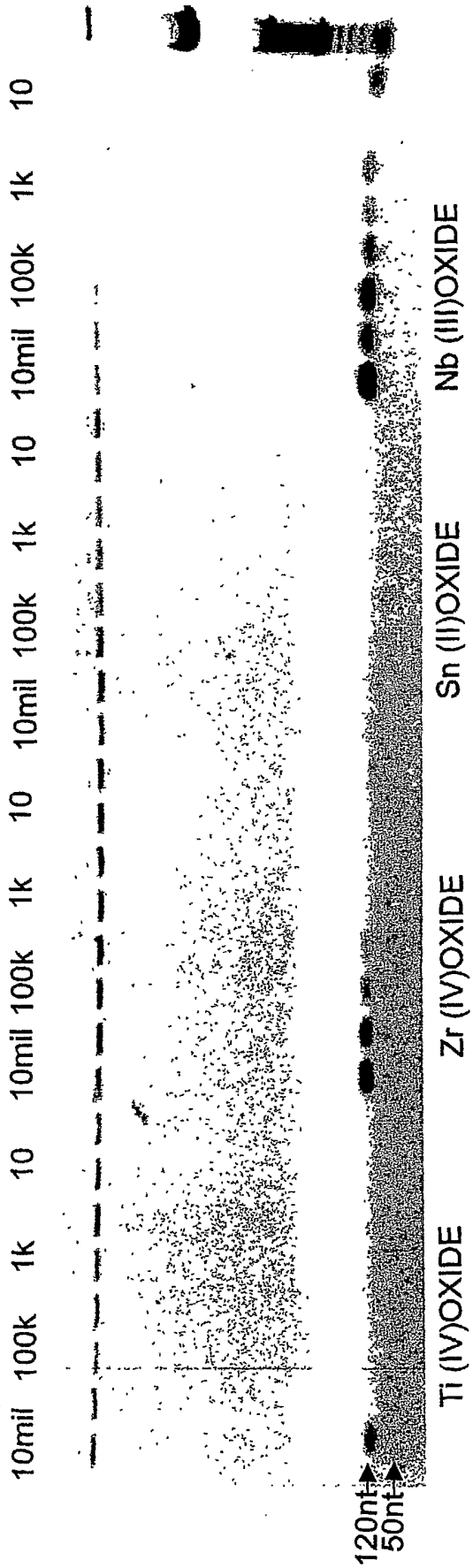
1mil
10k
1k
100
10
NTC
1mil
10k
100k
1k
NTC
1mil
10k
100k
1k
100
10
NTC
Al OXIDE
100MESH
Ti(III) OXIDE
Fe(III) OXIDE
120nt
50nt

FIG.1A


1mil 10k 100 NTC 1mil 10k 100 NTC 1mil 10k 100 NTC 1mil 10k 100 NTC
10mil 100k 1k 10 10mil 100k 1k 10 10mil 100k 1k 10 10mil 100k 1k 10
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Zr (IV)OXIDE
Sn (II)OXIDE
Nb (III)OXIDE
120nt
50nt

FIG. 1B

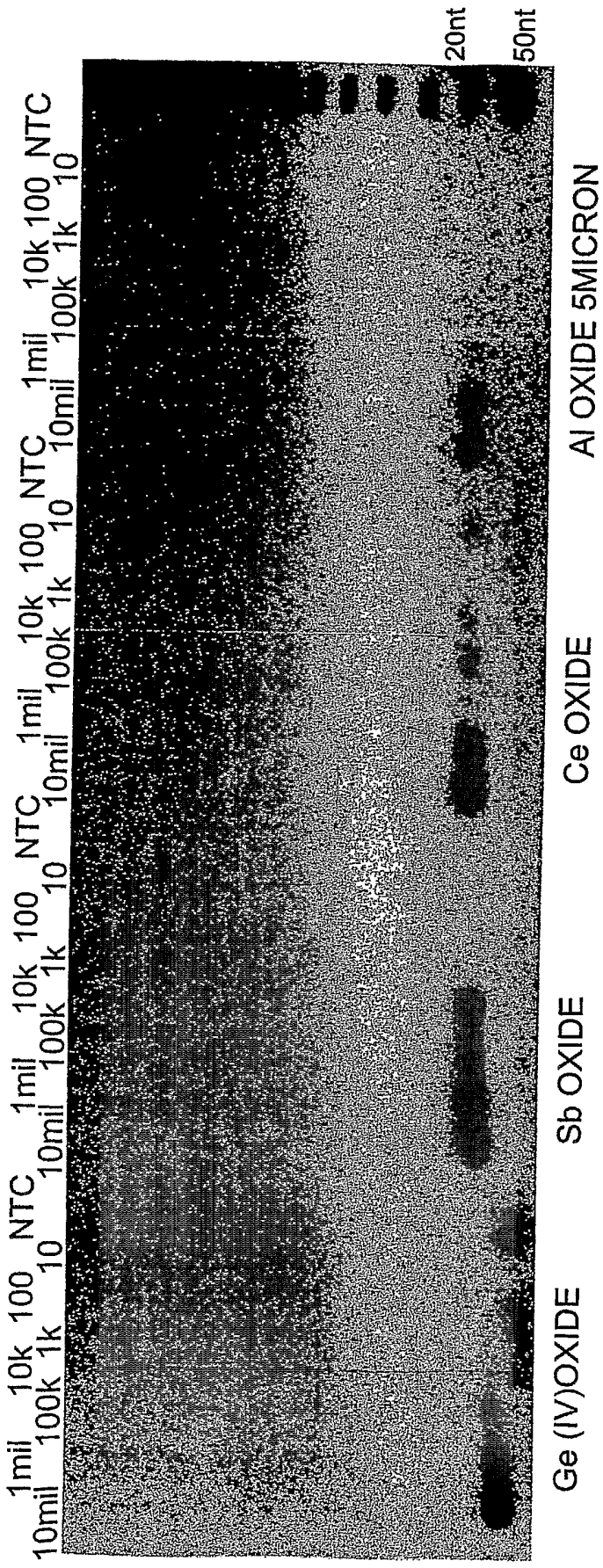
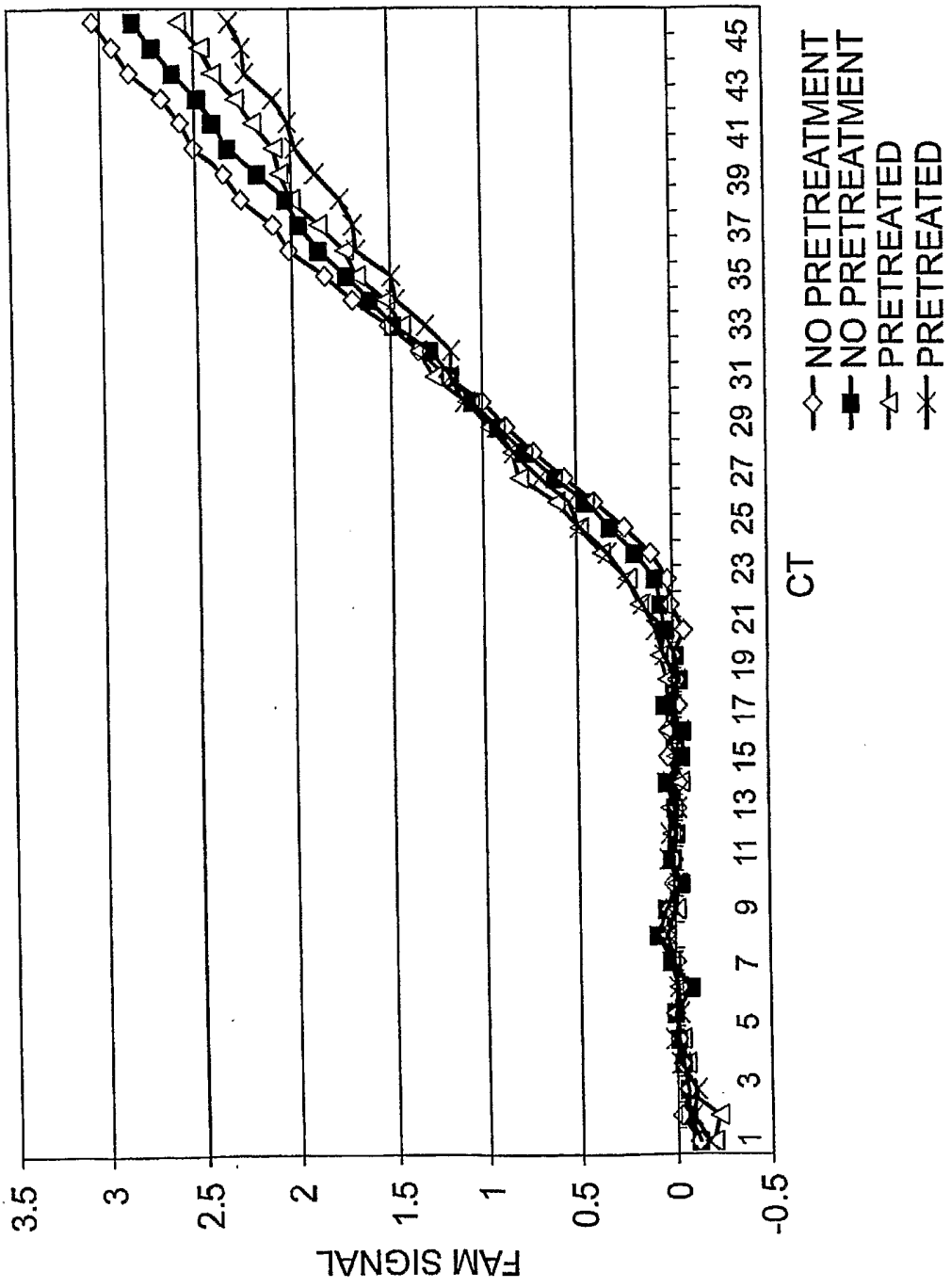

20nt
50nt
1mil 10mil 100k 1k 10k 100 10 NTC
Ge (IV)OXIDE
Sb OXIDE
Ce OXIDE
Al OXIDE 5MICRON

FIG. 1C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3
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2
1.5
1
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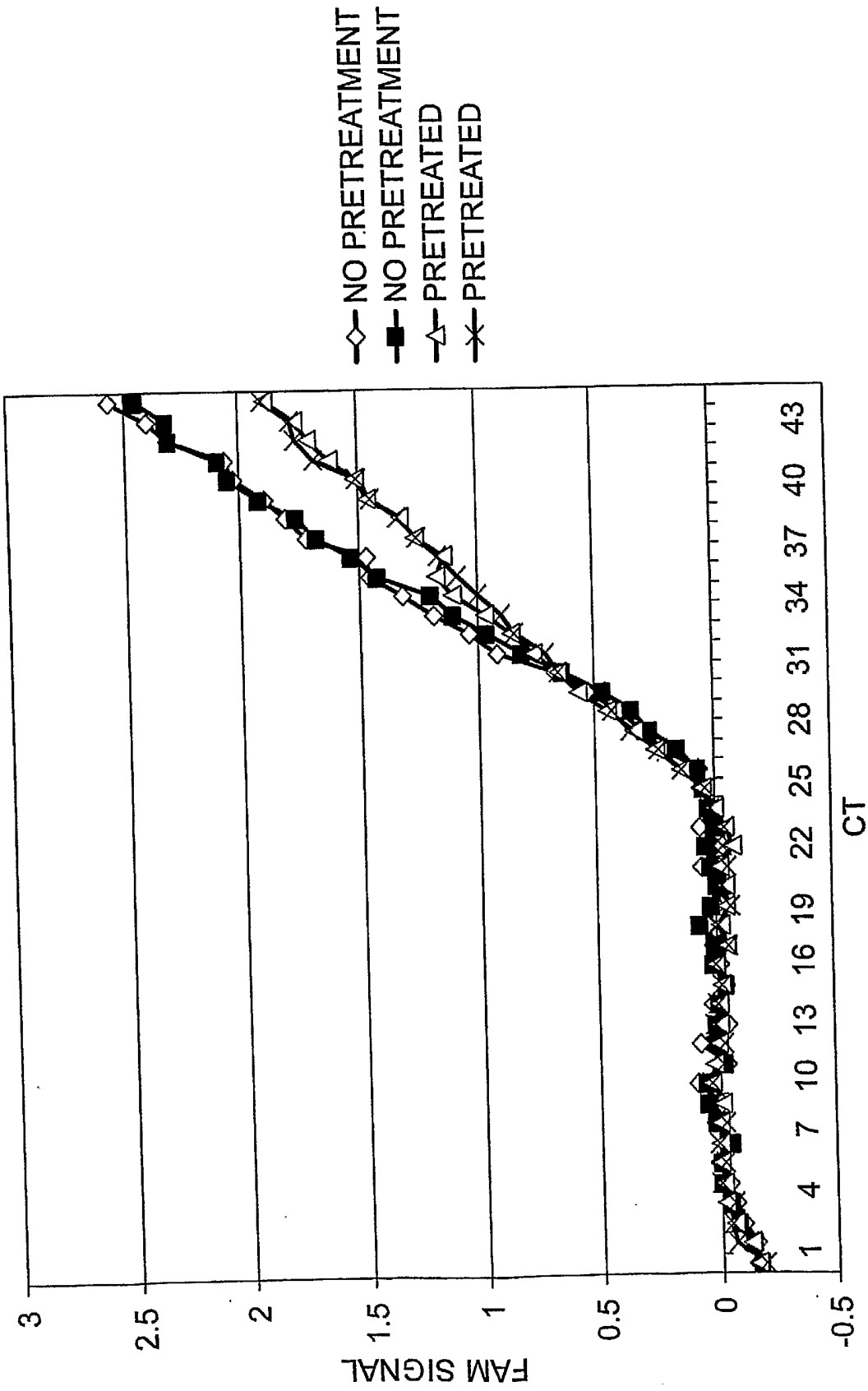
PRETREATED
PRETREATED

FIG. 2A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NO PRETREATMENT
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PRETREATED
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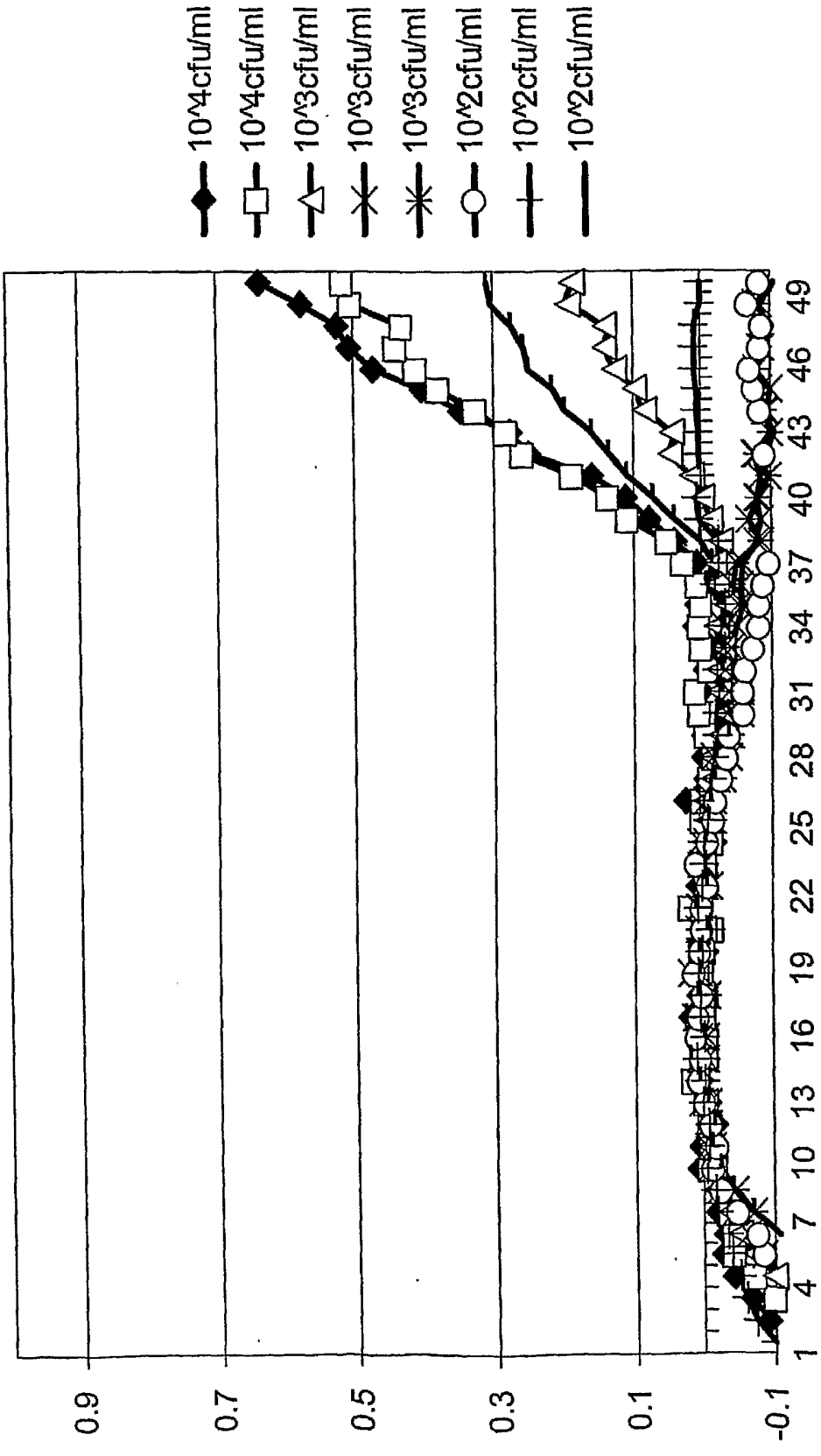
40
43
CT

FIG. 2B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0.9
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0.3
0.1
-0.1
FAM SIGNAL
1
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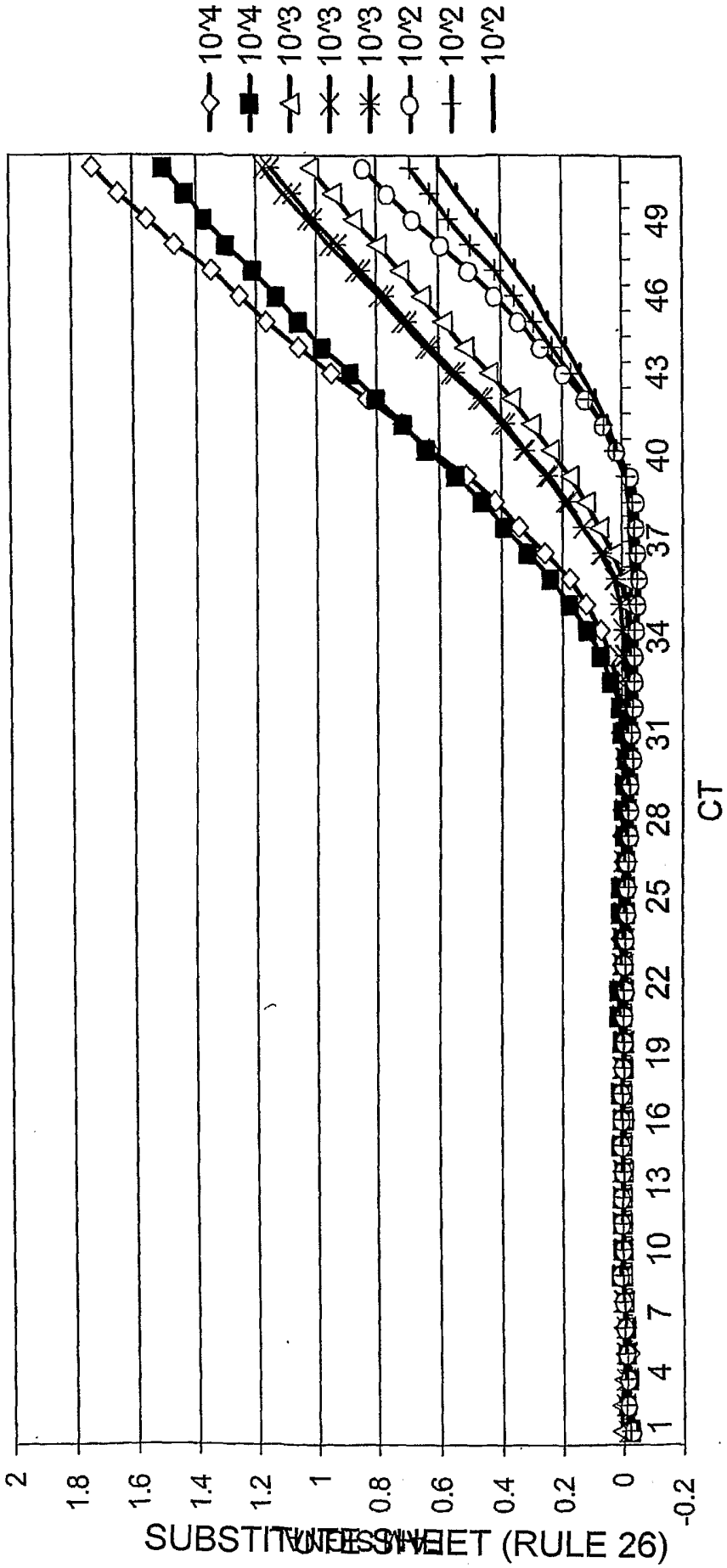
37
40
43
46
49
CT

FIG. 3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10^4
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10^3
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1
0.8
0.6
0.4
0.2
0
-0.2
1
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19
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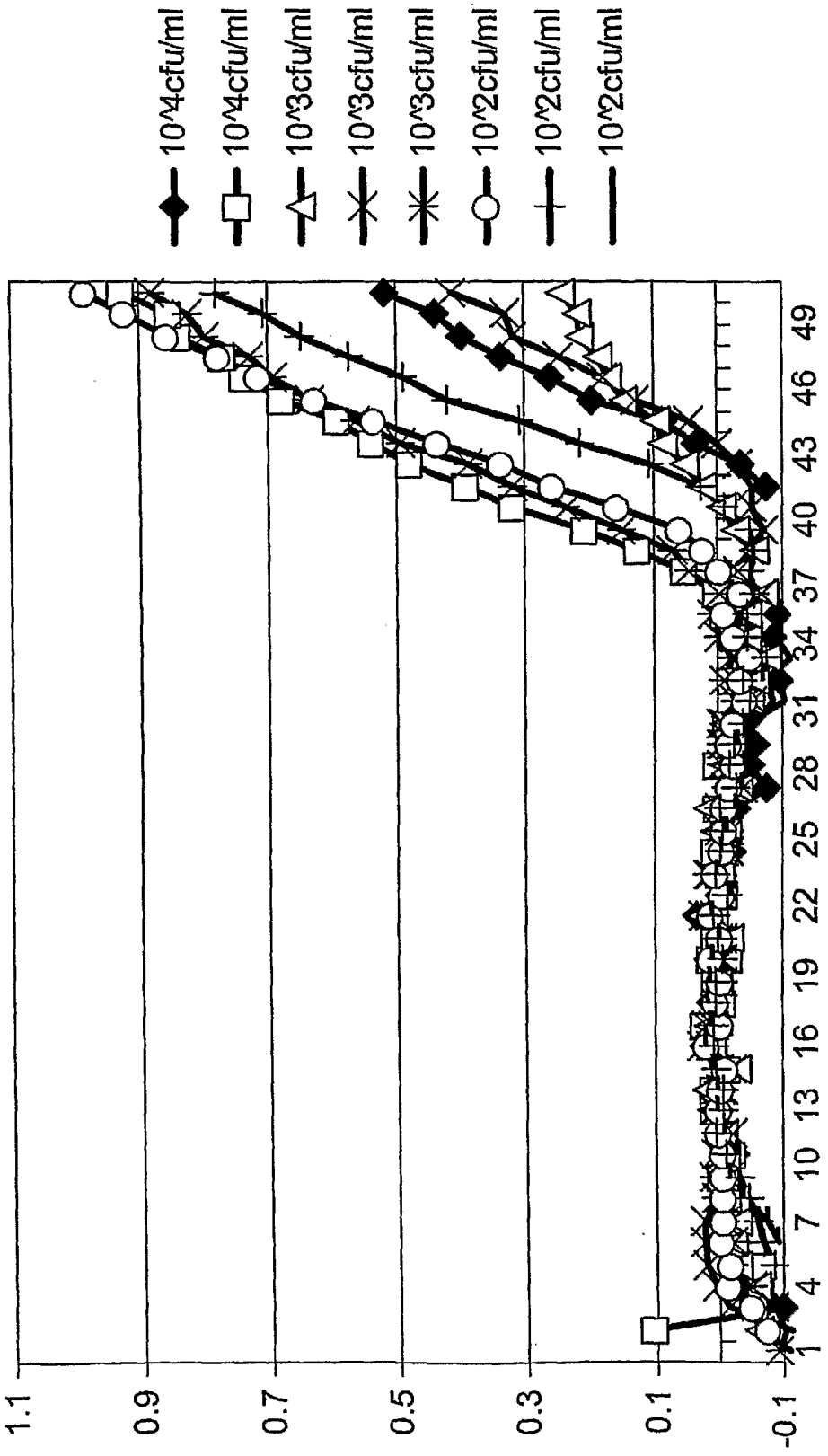
34
37
40
43
46
49
CT

FIG. 4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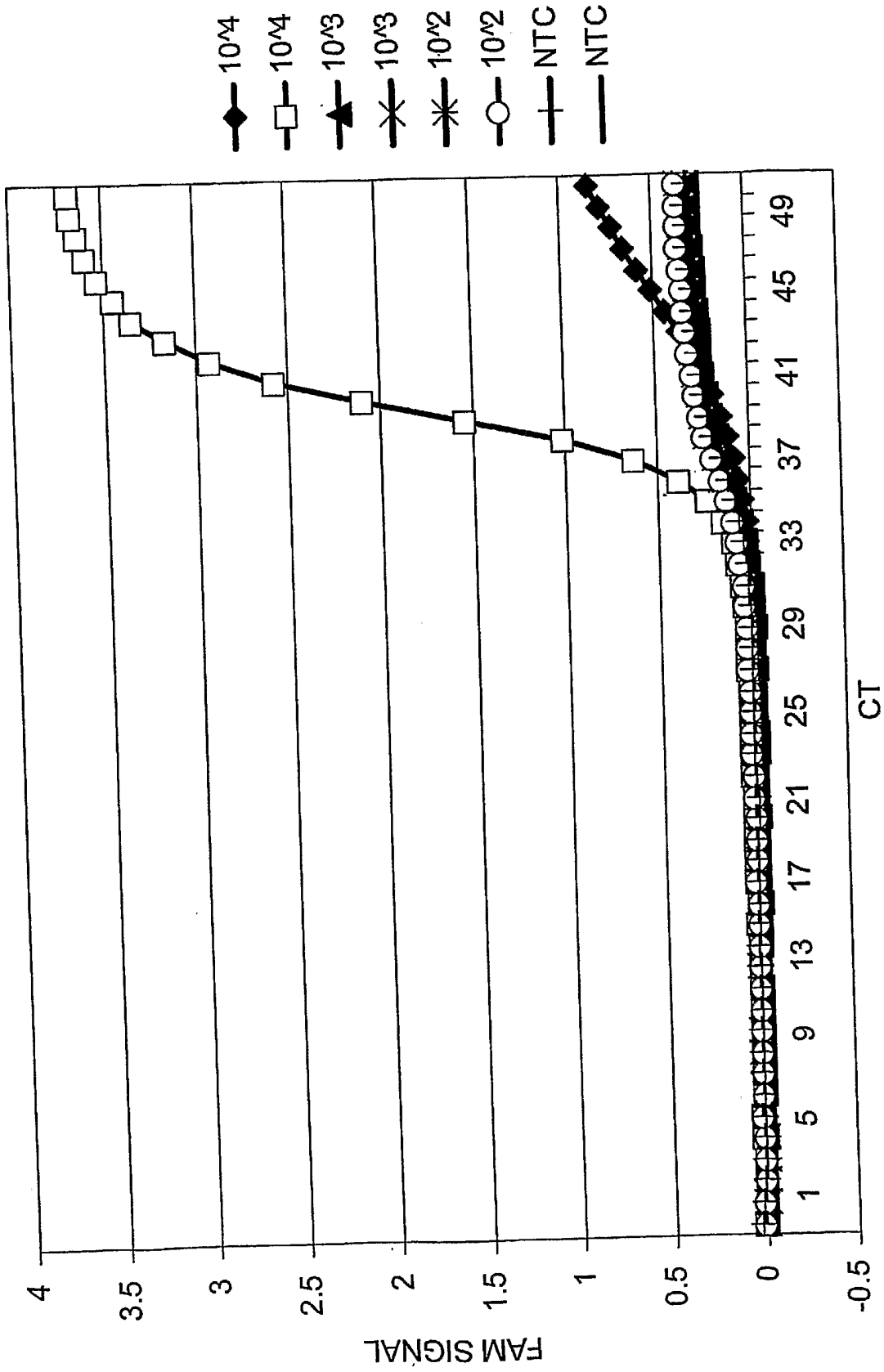
10^2cfu/ml
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
FAM SIGNAL
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
CT

FIG. 5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2
1.5
1
0.5
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CT
1
5
9
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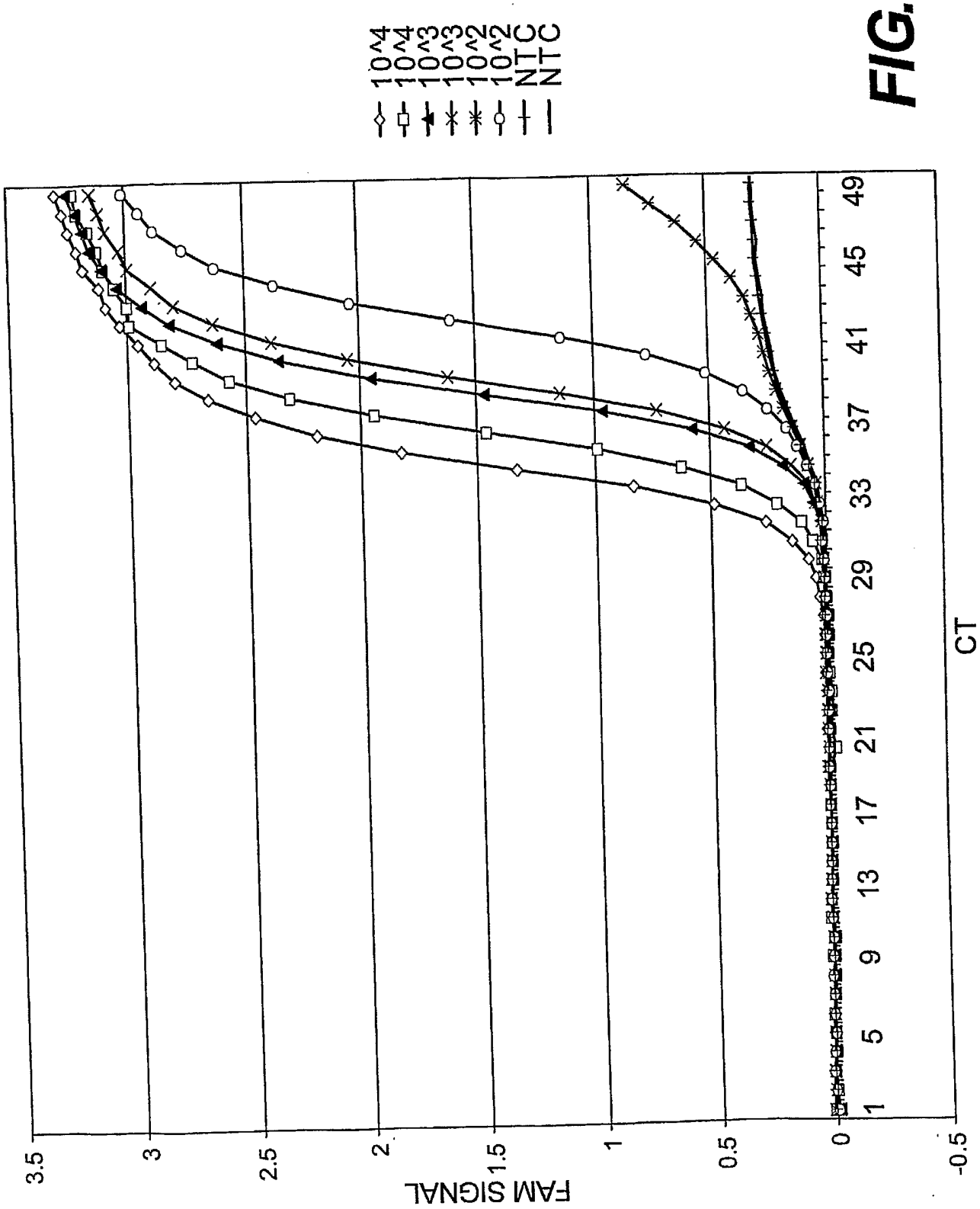
17
21
25
29
33
37
41
45
49

FIG. 6

FIG. 7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21
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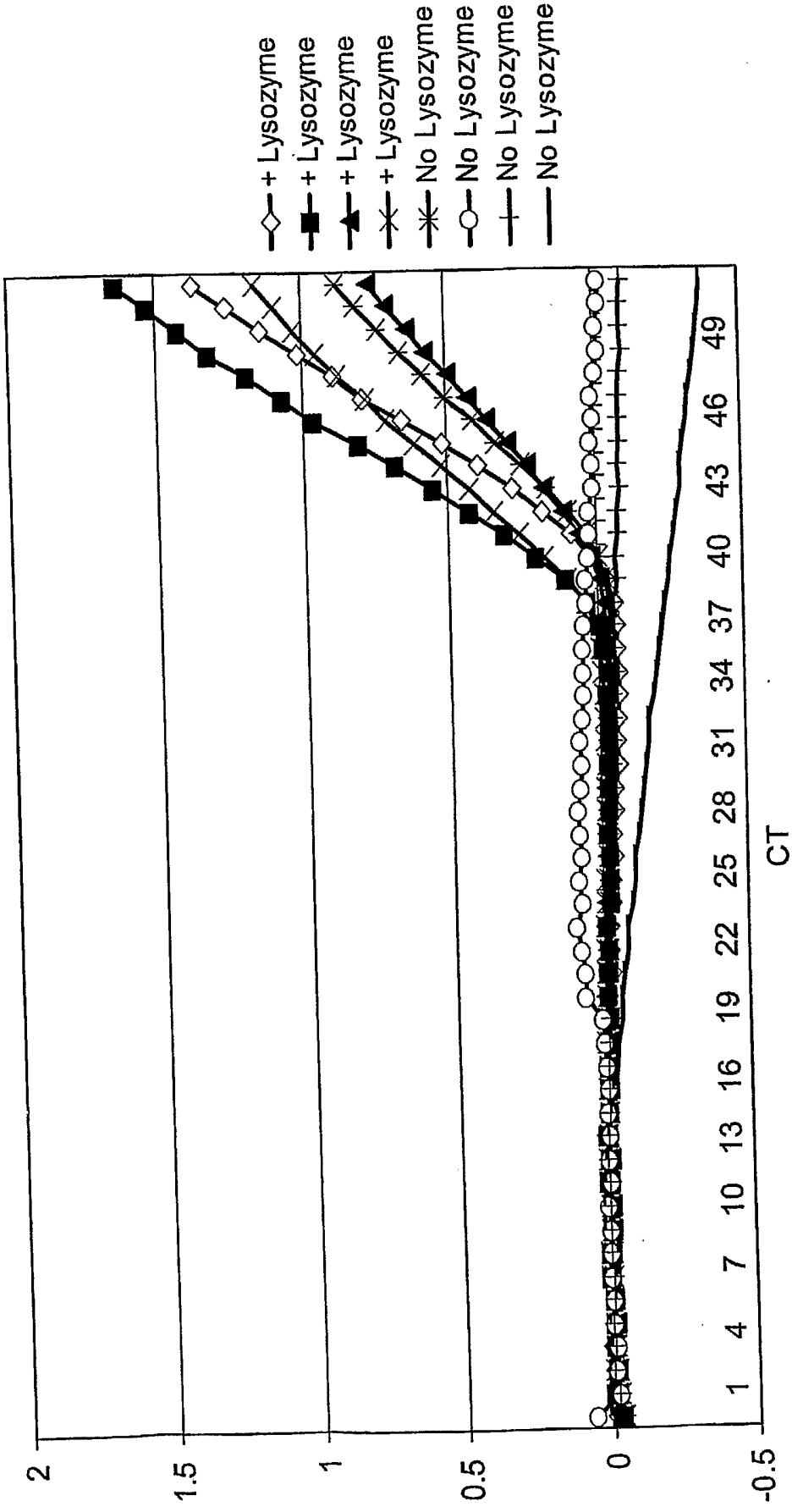
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+ Lysozyme
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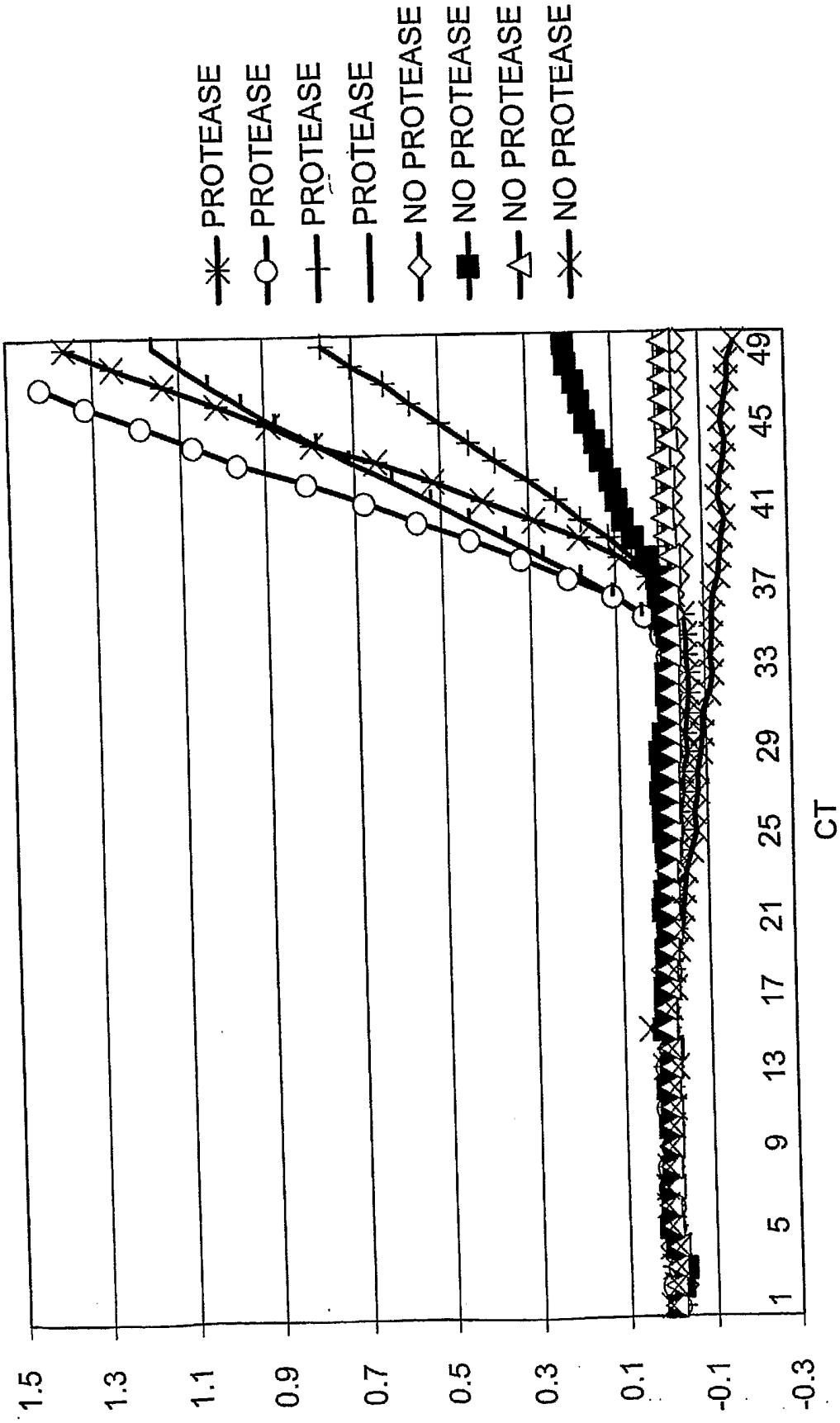
CT
FAM SIGNAL

FIG. 8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0.7
0.5
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0.1
-0.1
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5
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33
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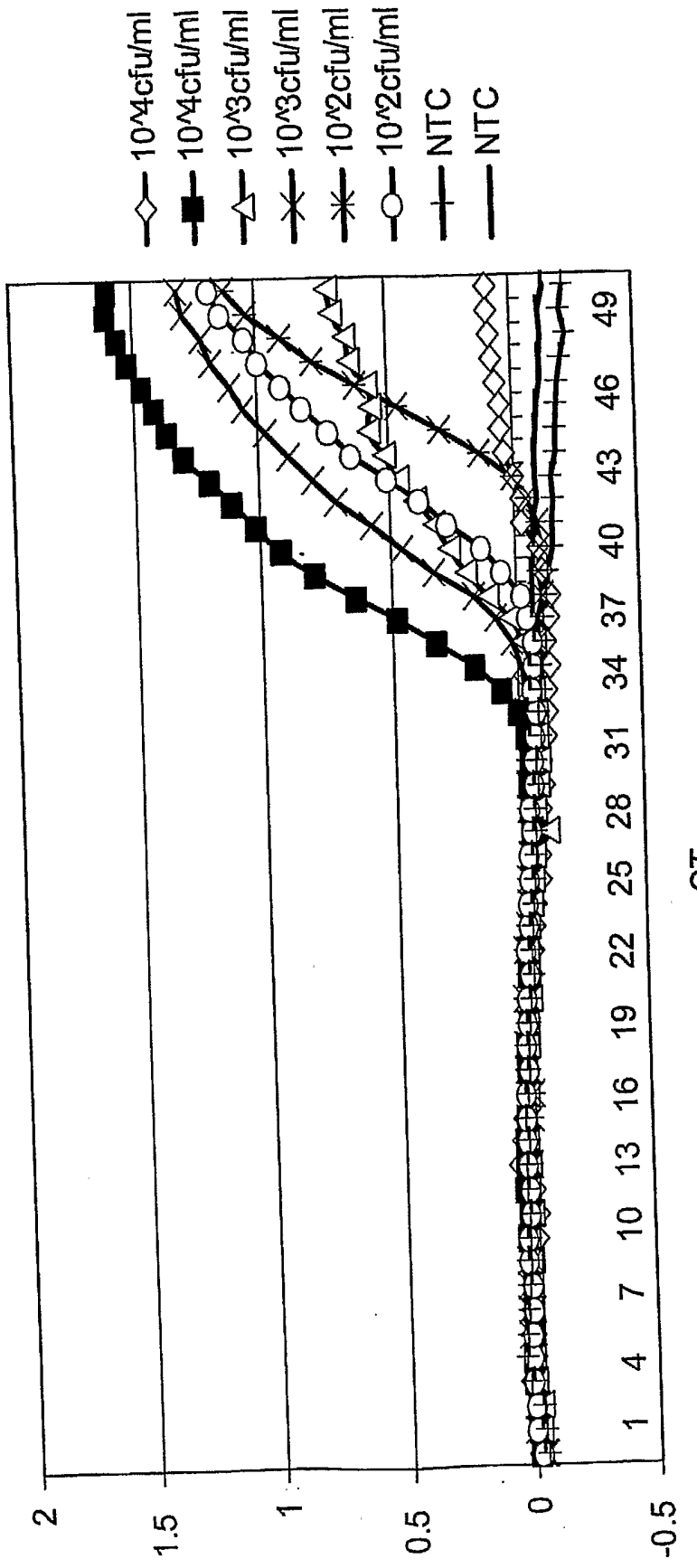
41
45
49
CT

FIG. 9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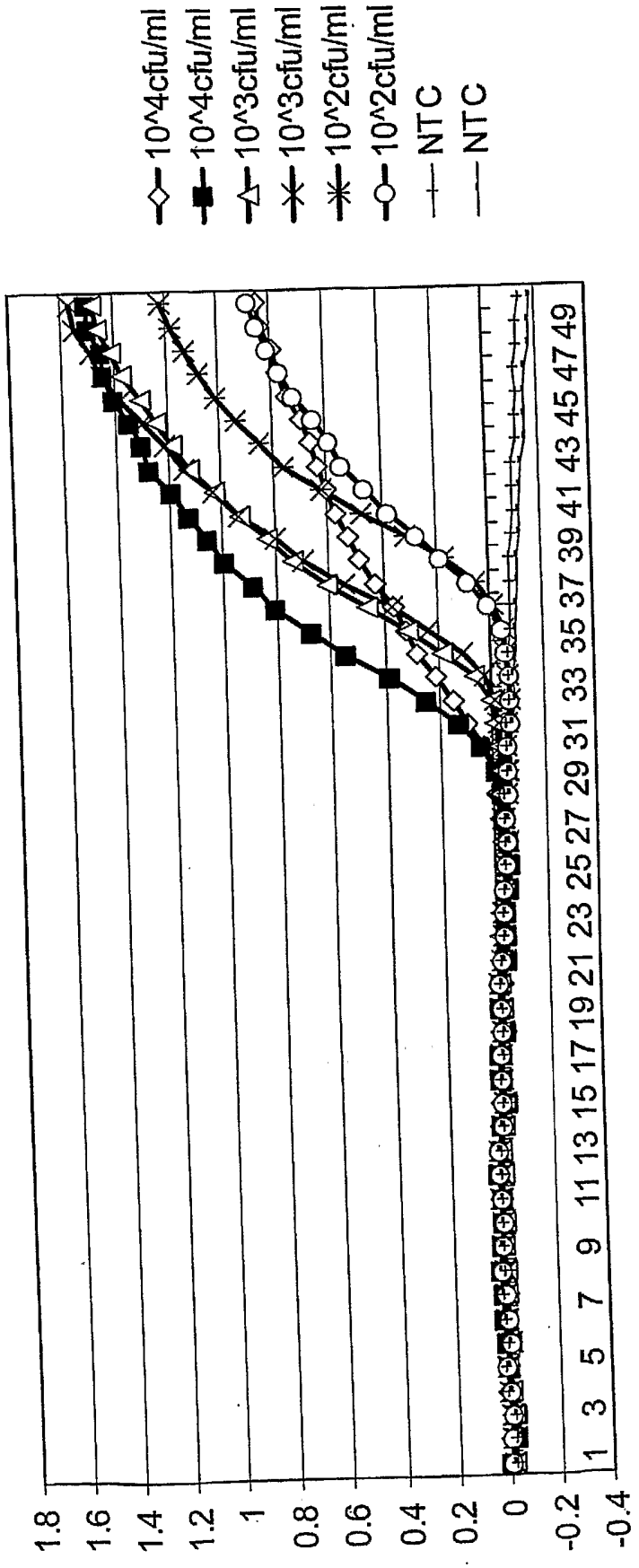
CT

FIG. 10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0.8
0.6
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FAM
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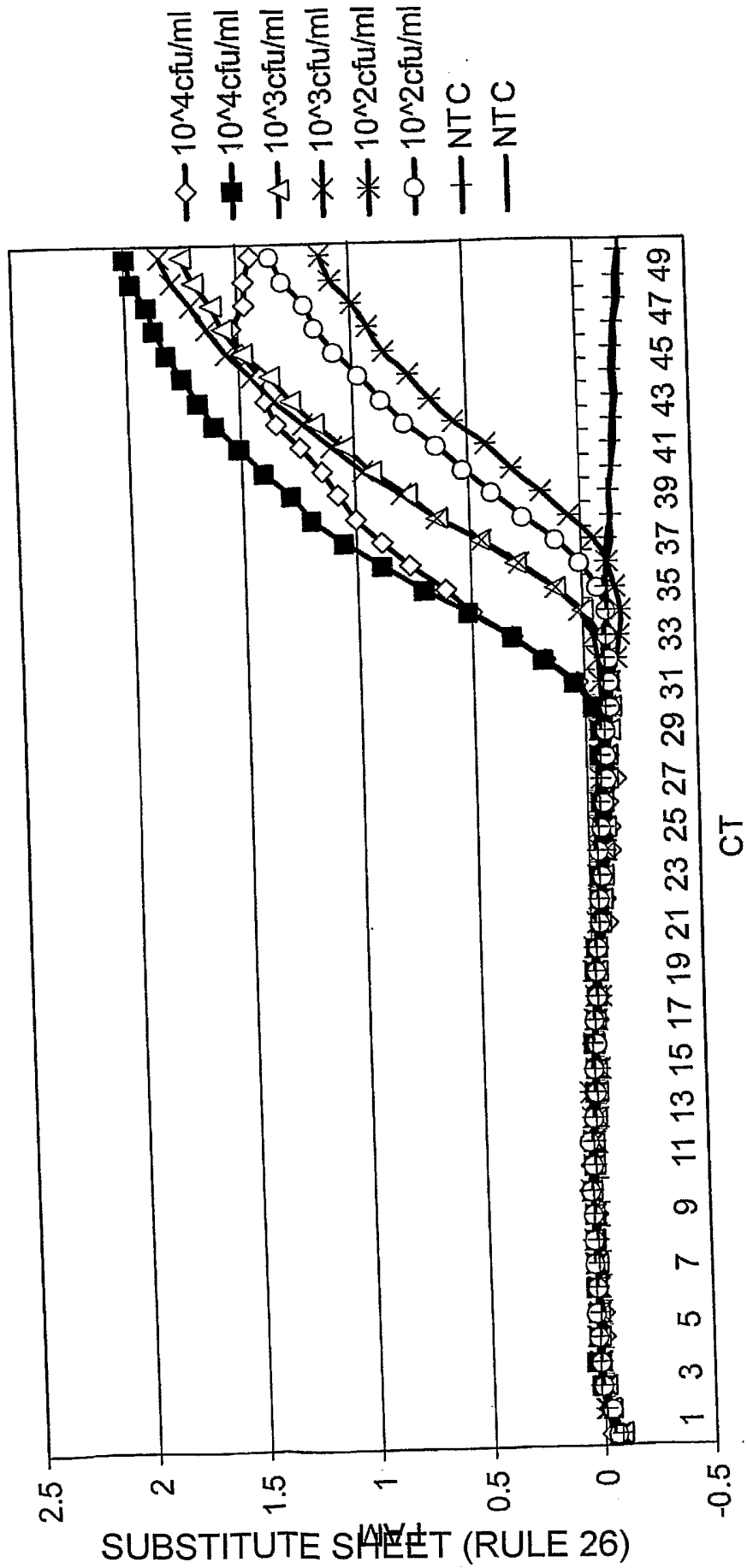
CT

FIG. 11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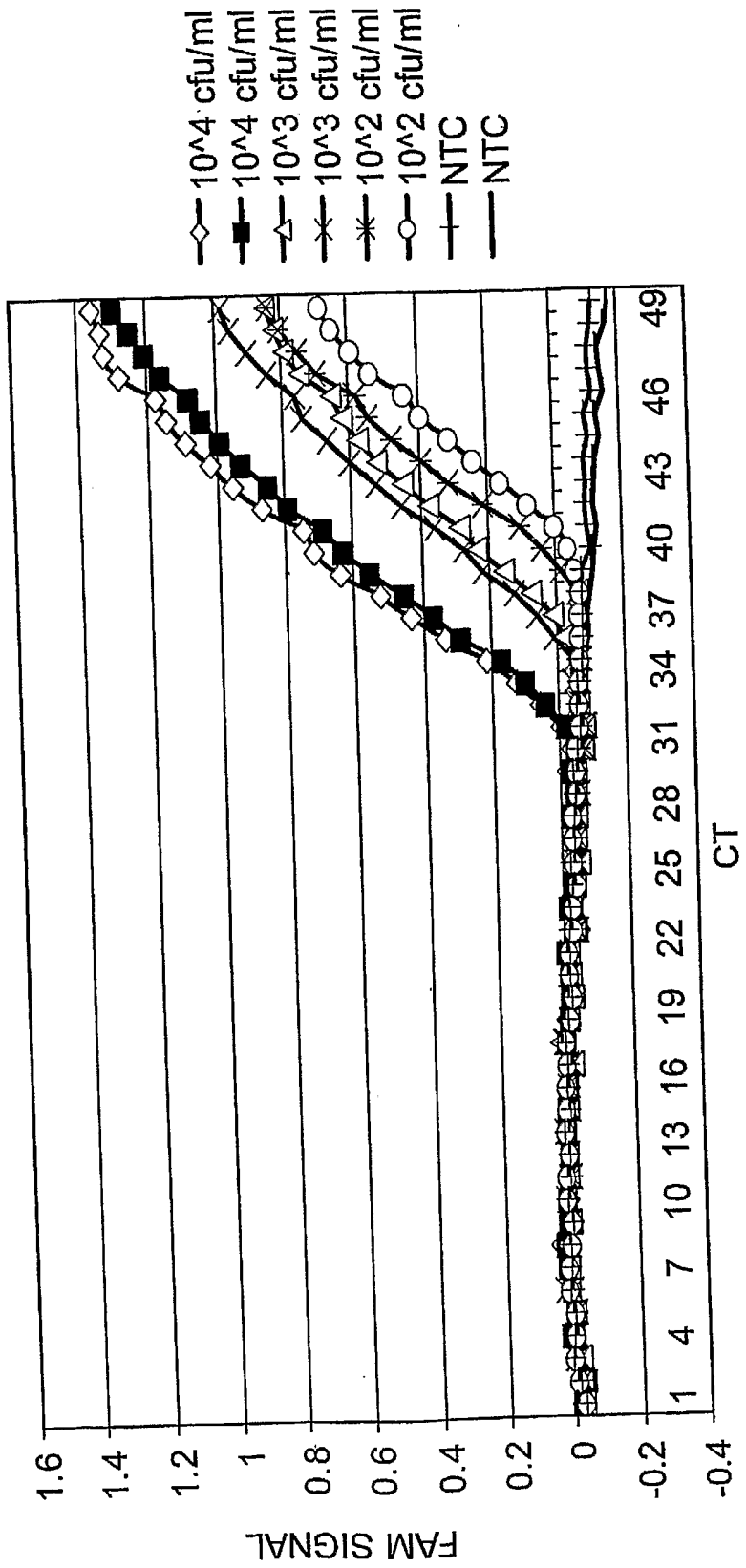
CT
ΔRn

FIG. 12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10^2 cfu/ml
NTC
NTC
FAM SIGNAL
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1.2
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0.8
0.6
0.4
0.2
0
-0.2
-0.4
CT
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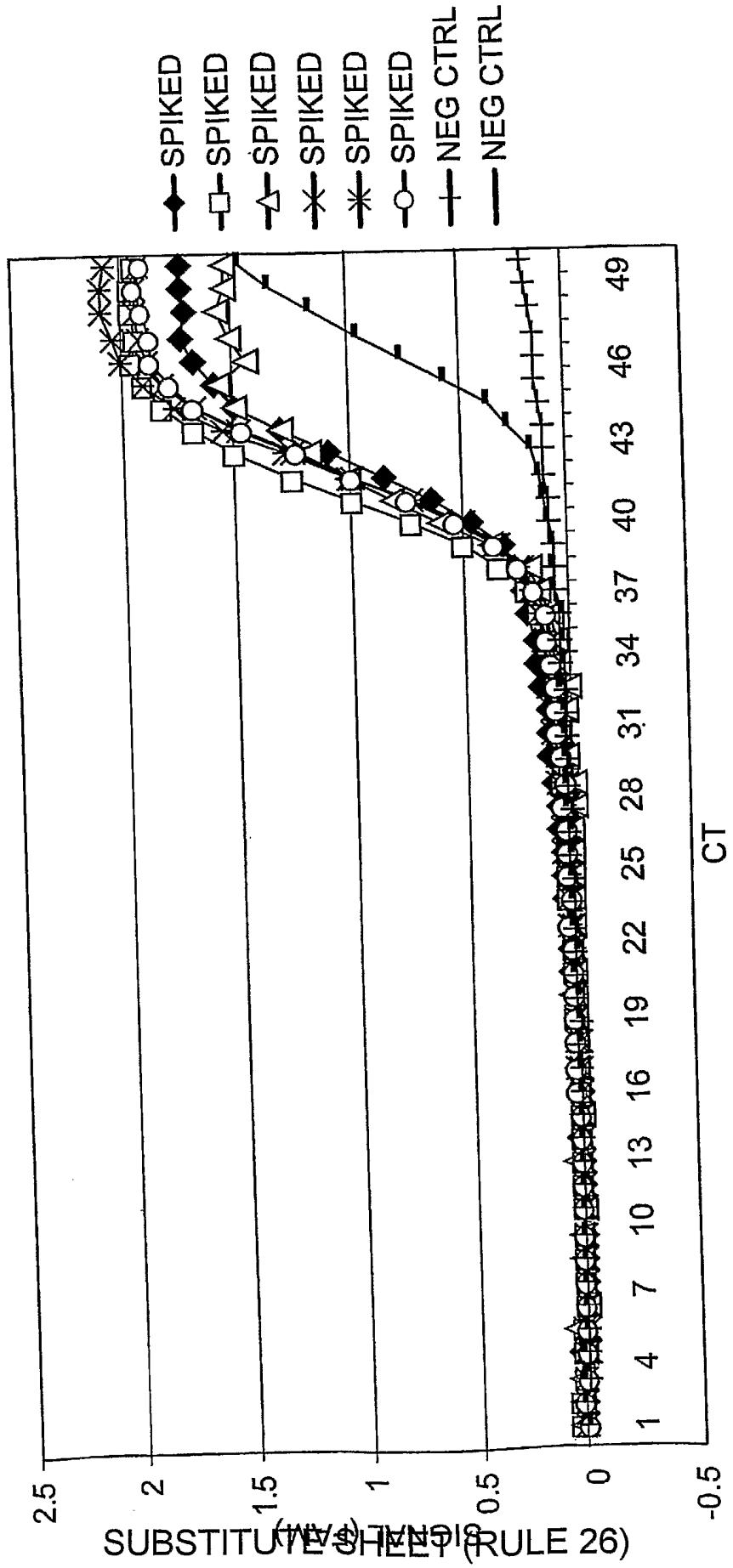
FIG. 13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NEG CTRL
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0
-0.5
1
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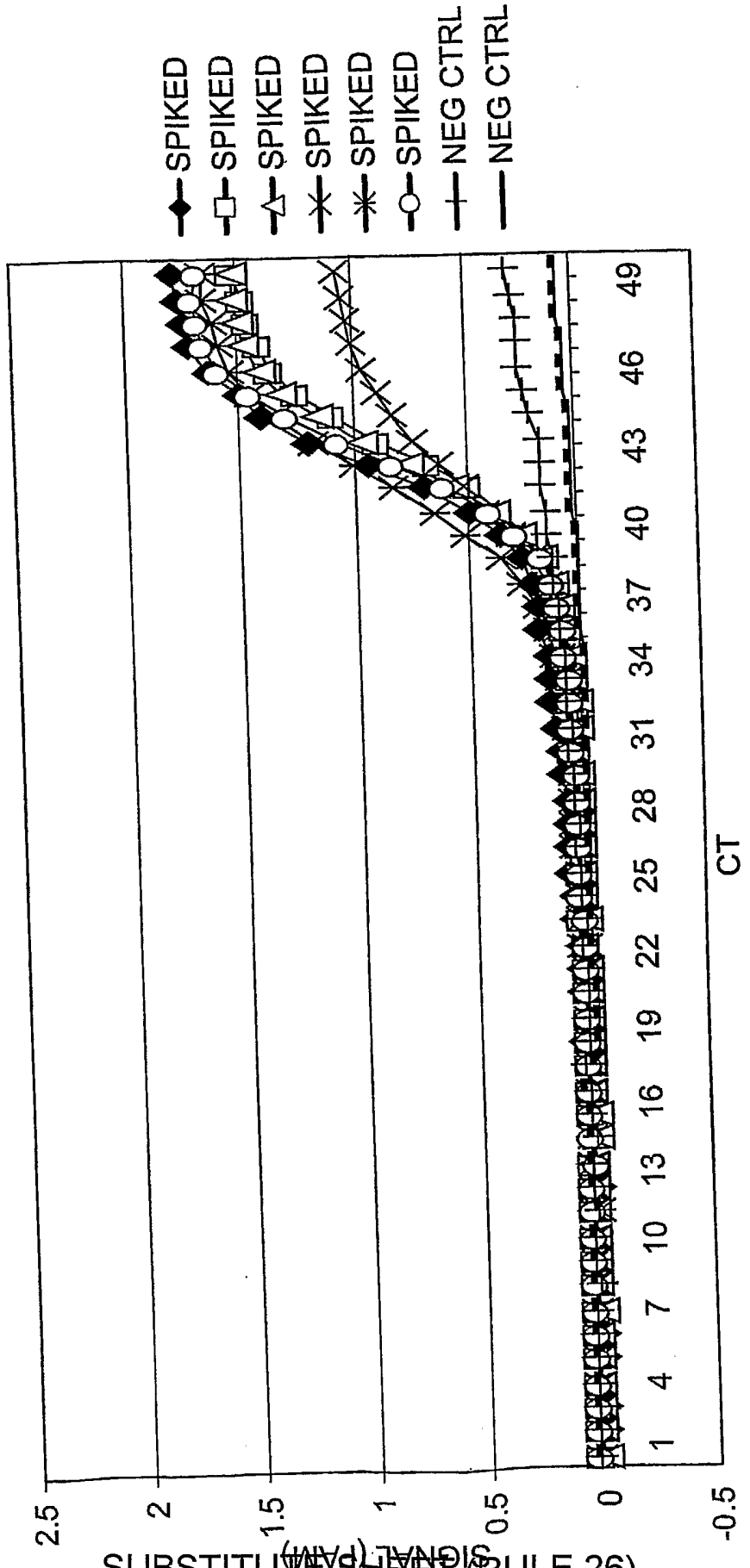
CT
SIGNAL (RFU)

FIG. 14A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SPIKED
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0
-0.5
SIGNAL (FAM)
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40
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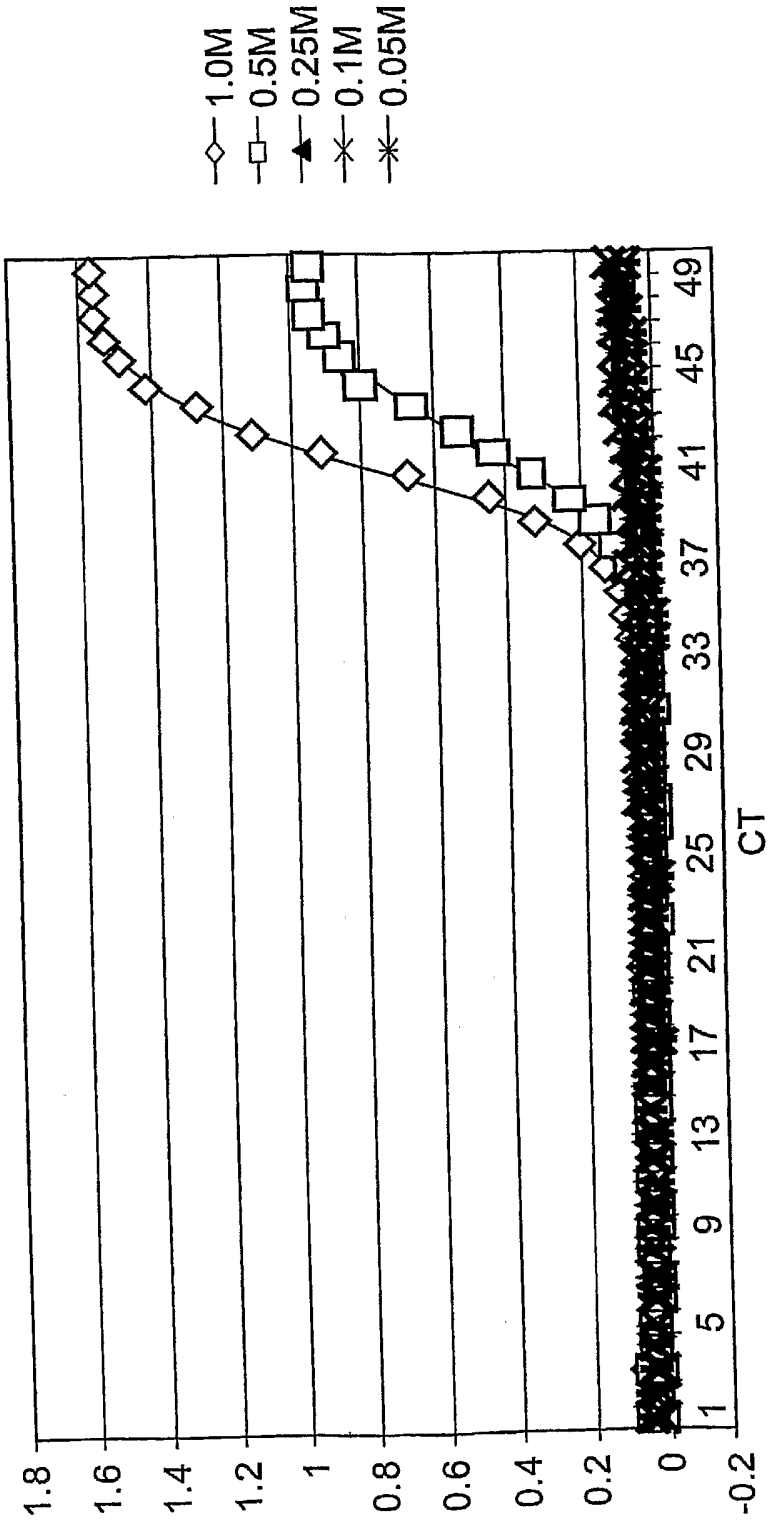
46
49
CT

FIG. 14B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0.1M
0.05M
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1.6
1.4
1.2
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0.8
0.6
0.4
0.2
0
-0.2
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SIGNAL (FAM)

FIG. 15

SEQUENCE LISTING

<110> WISNIEWSKI, MICHELE
BOYD, BRENDAN
BARBOUR, MARK

<120> METHODS, COMPOSITIONS, AND KITS FOR DETECTING NUCLEIC ACIDS IN A SINGLE VESSEL

<130> 09672.0004-00304

<150> US 11/096,775

<151> 2005-04-01

<160> 4

<170> PatentIn version 3.2

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tcactttagg tagtggttct acagttgctg gttatagtgc attatccgtt gctgatgctg 180

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caaaccaaac accgcaaatg ttaactcaat taattaacag ctaa 464

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/11554

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **C12P 19/34**(2006.01);**C12N 9/00**(2006.01);**C07H 21/04**(2006.01)

USPC: 435/91.2,183;536/24.3,24.32
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)
U.S. : 435/91.2,183;536/24.3,24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2004/0115663 A1 (BERLIN et al) 17 June 2004 (17.06.2004), see entire document.	1,7-9, 16-19, 22-31,33-35,39-45,49-67 --- 2-6, 10-15, 20-21, 32,36-38,46-48,68
X --- Y	US 2002/0132242 A1 (Gerdes et al) 19 September 2002 (19.09.2002), see entire document.	1,7-14,16-30,33-35,37, 39-45,47,49-66 --- 2-6,15,31,32,36,38,46,48, 67,68
Y	US 6,054,305 (Tatsumi et al) 25 April 2000 (25.04.2000), see column 5, lines 21-33.	2-6,15,36,38,46,48

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
20 July 2006 (20.07.2006)

Date of mailing of the international search report
28 AUG 2006

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/11554

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,866,336 (Nazarenko et al) 2 February 1999 (02.02.1999), see entire document.	1-68

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/11554

Continuation of B. FIELDS SEARCHED Item 3:
EAST, MEDLINE, STN
search terms: microorganism, vessel, nucleic acid, metal oxide, amplification, lysis, lysozyme, proteinase K, Tween-20, CTAB, Triton X-100