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Improved methods for determining cell viability using molecular nucleic acid-based techniques

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(56) Related Art
US 6,210,881 B1
WO 2009/082747 A1
WO 2002/052034 A1
WO 2007/100762 A2

AMENDMENT TO THE CLAIMS:

Please amend the claims as set forth below. No new matter has been added.

1. (Currently Amended) A method for ~~selectively excluding, from molecular detection, DNA of dead cells~~ detecting viable microbes in a [[from]] a mixture containing live and dead cells from a nucleic acid amplification assay thereby indicating that viable cells are present, comprising:
   a. removing dead microbe cell DNA prior to ~~obtaining a positive non contaminated result by~~ performing a nucleic acid amplification assay; ~~the steps of measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes,~~
   b. eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant; [[and]]
   c. performing the nucleic acid amplification assay, wherein obtaining a positive non contaminated result from the assay indicates that viable cells are present; and
   d. ~~determining the ratio of live to dead microbes present in the mixture~~ measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes.

2. (Canceled) ~~In a method for the determination of the ratio of live to dead microbes present in a mixture containing live and dead cells that can be used as a measure of the effectiveness of therapy or efficacy of a treatment by performing a nucleic acid amplification assay thereby indicating that viable cells are present, the improvement comprising measuring tool more time points of microbe-specific signal increases form the amplification assay as an indication of the presence of viable microbes, eliminating amplification assay inhibitors form the mixture by the addition of a chemical denaturant, and determining the ratio of lives to dead microbes present in the mixture~~.

3. (Original) The method of claim 1, wherein a chemical denaturant comprises a mixture of one or more chemical agents.

4. (Original) The method of claim 1, wherein the amplification assay is a PCR assay.

5. (Original) The method of claim 1, wherein the mixture comprises blood and other body fluids.

6. (Currently Amended) The method of claim 4, ~~wherein~~ further comprising performing the PCR assay to thereby obtain ~~provides~~ in addition a correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis and septicemia.

7. (Canceled) ~~The method of claim 1, wherein signals from killed cells in the mixture are suppressed and membrane-compromised cells in the mixture are excluded from analysis.~~

8. (New) The method of claim 4, wherein the PCR assay is a qPCR assay.

REMARKS

Claims 1-8 are pending in the application with claims 1 being independent. Claim 8 is newly added. Claims 2 and 7are canceled.

Claim 2 is objected to for containing numerous grammatical errors.

Claims 1-7 are rejected under 35 U.S.C. §112(b) as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or joint inventor, all for pre-AIA the applicant regards as the intention.

Claims 2-7 are rejected solely for being dependent on claim 1.

Claims 1 and 2 are vague and indefinite because it is unclear what order should the recited steps be performed in because the step of measuring time points of microbe-specific signal with an application assay is followed ID addition of chemical denaturant to eliminate amplification assay inhibitors. As recited, it is unclear how the artisan is to measure microbe specific signals with an amplification assay prior to eliminating the inhibitors of an amplification assay by adding of a chemical denaturant.

Claims 3-7 are rejected as indefinite because they depend from claim 1.

Claims 6 is indefinite as it is unclear whether septicemia detection is required or whether the claim is reciting an inherent property of the method.

Claim 6 is rejected under 35 U.S.C. §112(d) or pre-AIA 35 U.S.C. 112 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, all for failing to include all the limitations of the claim upon which it depends.

Claims 6 recites the limitation "wherein performing the PCR assay provides in addition a correlation with viable microbe cells from the Bacteremia and Fungemia samples for the diagnosis of septicemia." It is unclear whether claim six requires septicemia detection of if an inherent capability all intended use of the method is recited. If the claim is simply reciting an intended use or inherit property, it is not further limiting.

Claims 1-7 are rejected under pre-AIA 35 U.S.C. §103(a) as being unpatentable over Nocker et al. (Mar 2009, J. Microbiol Methods, 76(3):253-61. Epub 2008 Dec 7:previously cited) in view of Nocker et al. (2007, J. Microbiol. 70(2):252-60. Epub 2007 May 1: previously cited) and Gebert et al. (2008, J. Infect. 57(4):307-16. Epub 2008 Aug

29: previously cited) as evidenced by Horz et al. (2008 Jan; 72(1): 98-102) and McCann et al. (Apr. 2014, J. Microbiol Methods, 99:1-7. Epub 2014 Feb 3:previously cited).

Each of the various rejections and objections are overcome by amendments that are made to the specification, drawing, and/or claims, as well as, or in the alternative, by various arguments that are presented.

Entry of this Amendment is proper under 37 CFR §1.116 since the amendment: (a) places the application in condition for allowance for the reasons discussed herein; (b) does not raise any new issue requiring further search and/or consideration since the amendments amplify issues previously discussed throughout prosecution; (c) satisfies a requirement of form asserted in the previous Office Action; (d) does not present any additional claims without canceling a corresponding number of finally rejected claims; or (e) places the application in better form for appeal, should an appeal be necessary. The amendment is necessary and was not earlier presented because it is made in response to arguments raised in the final rejection. Entry of the amendment is thus respectfully requested.

Any amendments to any claim for reasons other than as expressly recited herein as being for the purpose of distinguishing such claim from known prior art are not being made with an intent to change in any way the literal scope of such claims or the range of equivalents for such claims. They are being made simply to present language that is better in conformance with the form requirements of Title 35 of the United States Code or is simply clearer and easier to understand than the originally presented language. Any amendments to any claim expressly made in order to distinguish such claim from known prior art are being made only with an intent to change the literal scope of such claim in the most minimal way, i.e., just to avoid the prior art in a way that leaves the claim novel and not obvious in view of the cited prior art, and no equivalent of any subject matter remaining in the claim is intended to be surrendered.

Also, because a dependent claim inherently includes the recitations of the claim or chain of claims from which it depends, it is submitted that the scope and content of any dependent claims that have been herein rewritten in independent form is exactly the same as the scope and content of those claims prior to having been rewritten in independent form. That is, although by convention such rewritten claims are labeled herein as having

been "amended," it is submitted that only the format, and not the content, of these claims has been changed. This is true whether a dependent claim has been rewritten to expressly include the limitations of those claims on which it formerly depended or whether an independent claim has been rewritten to include the limitations of claims that previously depended from it. Thus, by such rewriting no equivalent of any subject matter of the original dependent claim is intended to be surrendered. If the Examiner is of a different view, he is respectfully requested to so indicate.

Rejection Under 35 U.S.C. §112(b)

Claim 2 is objected to for containing numerous grammatical errors. Claim 2 is canceled; therefore the objection is moot. As a result, the objection should be withdrawn.

Claims 1-7 are rejected under 35 U.S.C. §112(b) as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or joint inventor, all for pre-AIA the applicant regards as the intention.

The amendment to claim 1 obviates the rejection. Specifically, claim 1 now recites the steps of the method in the expected order the Examiner referred to. As a result, the rejection should be withdrawn.

Claims 2-7 are rejected solely for being dependent on claim 1. As indicated above, the amendment to claim 1 renders this rejection moot. As a result, the rejection should be withdrawn.

Claims 1 and 2 are vague and indefinite. Again, the amendment to claim 1 renders this rejection moot. As a result, the rejection should be withdrawn.

Claims 3-7 are rejected as indefinite because they depend from claim 1. Once again, the amendment to claim 1 renders this rejection moot. As a result, the rejection should be withdrawn.

Claims 6 is indefinite as it is unclear whether septicemia detection is required or whether the claim is reciting an inherent property of the method. Claim 6 is amended to address the Examiner's rejection. Withdrawal of the rejection is requested.

Rejection Under 35 U.S.C. §112(d)

Claim 6 is rejected under 35 U.S.C. §112(d) or pre-AIA 35 U.S.C. 112 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, all for failing to include all the limitations of the claim upon which it depends.

As indicated above, claim 6 is amended to further limit claim 4 from which it depends. Withdrawal of the rejection is requested.

Rejection Under 35 U.S.C. §103

The Examiner bears the initial burden of establishing a prima facie case of obviousness. See MPEP § 2141. Establishing a prima facie case of obviousness begins with first resolving the factual inquiries of Graham v. John Deere Co. 383 U.S. 1 (1966). The factual inquiries are as follows:

(A) determining the scope and content of the prior art;

(B) ascertaining the differences between the claimed invention and the prior art;

(C) resolving the level of ordinary skill in the art; and

(D) considering any objective indicia of nonobviousness.

Once the Graham factual inquiries are resolved, the Examiner must determine whether the claimed invention would have been obvious to one of ordinary skill in the art. The key to supporting a rejection under 35 U.S.C. §103 is the clear articulation of the reasons why the claimed invention would have been obvious. The analysis supporting such a rejection must be explicit. "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." In re Kahn, 441 F. 3d 977, 988 (CA Fed. 2006), cited with approval in KSR Int'l Co. v. Teleflex, Inc., 126 S. Ct. 2965 (2006); see also MPEP §2141.

According to MPEP §2143.03: "All words in a claim must be considered in judging the patentability of that claim against the prior art" (*quoting*, *In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970)). In addition, to establish a prima facie case of obviousness the prior art reference (or references when combined) must teach or suggest all elements of the subject claim. *In re Wada*, 2007-3733 (BPAI Jan. 14, 2008) (*citing*, *CMFT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed.Cir. 2003)).

Claims 1-7 are rejected under 35 U.S.C. §103(a) as being unpatentable over Nocker, in view of Gebert and further in view of McCann. The rejection is traversed.

The Office Action fails to establish a *prima facie* case of obviousness, because the suggested combination of the references does not teach all of the elements of each of the amended independent claims. According to MPEP §2143, to establish a *prima facie* case of obviousness under §103, the prior art reference (or references when combined) must teach or suggest all the claim limitations. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). More specifically, claim 1 is amended to recite in part:

> "performing the nucleic acid amplification assay, wherein obtaining a positive non contaminated result from the assay indicates that viable cells are present; and
> measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes. " (emphasis added).

The above limitations are neither recited nor suggested by the prior art combined or alone. As such, independent claim 1 is allowable under 35 U.S.C. §103(a) over Nocker in view of Gebert and further in view of McCann.

Nocker, Gebert and McCann, alone or in any permissible combination, fail to teach or suggest all elements of independent claim 1. More specifically, it appears that the references individually or in combination do not teach at least the following feature recited in the claims: "performing the nucleic acid amplification assay, wherein obtaining a positive non contaminated result from the assay indicates that viable cells are present" (emphasis added).

Therefore, independent claim 1 is allowable over Nocker, Gebert and McCann under 35 U.S.C. §103(a). Because all of the dependent claims that depend from the independent claims include all the limitations of the respective independent claim from which they ultimately depend, each such dependent claim is also allowable over Nocker, Gebert and McCann under 35 U.S.C. §103(a).

Accordingly, because the cited references do not describe each element of Applicants' claim 1 with sufficient specificity, a *prima facie* case of obviousness regarding claim 1 has not been established.

Conclusion

It is respectfully submitted that the Office Action's rejections have been overcome and that this application is now in condition for allowance. Reconsideration and allowance are, therefore, respectfully solicited.

If, however, the Examiner still believes that there are unresolved issues, s/he is invited to call Applicants' attorney Emmanuel Coffy at (973) 375-1804 so that arrangements may be made to discuss and resolve any such issues.

Respectfully submitted,

/EC/

Dated:6/26/2015

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1637	

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12/26/2014	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 13/977,719	Applicant(s) O'HARA, SHAWN MARK	
	Examiner OLAYINKA OYEYEMI	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *11/21/2014*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-7* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-7* is/are rejected.

8)☒ Claim(s) *2* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *11/19/2014*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Status of the Applications, Amendments and/or Claims

1. This action is written in response to applicant's correspondence submitted November 21, 2014. In the paper of November 21, 2014, Applicants amended claims 1-2 and 6.

All claim amendments and arguments from the paper of November 21, 2014 have been thoroughly reviewed but were found insufficient to place the instantly examined claims in condition for allowance. The following rejections are either newly presented, as necessitated by amendment, or are reiterated from the previous Office Action. Any rejections not reiterated in this action have been withdrawn as necessitated by applicant's amendments to the claims. This Office action is **Final.**

Status of the Claims

2. Claims 1-7 are pending.

Response to Arguments

Objections

3. The objection to claim 1 for being grammatically incorrect is **withdrawn** based on the amendment of claim 1 to newly recite a thereby clause and other additional limitations. Applicant's argument (see page 4 of the Remarks of 11/21/2013, 1st para), that this objection has been obviated by amendment to claim 1 **is persuasive**.

Rejections

4. The rejection of claims 1-7 under 35 U.S.C. § 112(b), stated in the previous Office action of 05/21/2014 (pg 2-3) is **maintained** despite the amendment of claims 1, 2 and 6. The amendments made to claims 1 and 2 do NOT clarify the order of execution of the claimed method steps. The amendment made to claim 6 do not clarify whether the recited limitations of the claims as noted in the 112(b) rejection, are inherent or are intended use limitations.

5. The rejection of claim 2 under 35 U.S.C. § 112(d), stated in the previous Office action of

05/21/2014 (pg 3-4) is **withdrawn**.

6. The rejection of claim 6 under 35 U.S.C. § 112(d), stated in the previous Office action of 05/21/2014 (pg 3-4) is **maintained** despite the amendment to claim 6. The amendments made to claim 6 do not clarify whether the recited limitations of the claims as noted in the 112(d) rejection are inherent or are intended use limitations. Therefore, it is unclear whether the limitations noted in the rejection are further limiting of claim 1.

7. The rejection of claims 1-7 under 35 U.S.C. § 103(a), as stated in the previous Office action of 05/21/2014 (pg 4-10) is **maintained**. Applicant's arguments (see Remarks of 11/21/2014, pg 4, last para and pg 5, 1st-4th para), are addressed in the section entitled "Arguments" below.

Arguments

8. Applicant's arguments filed 11/21/2014 have been fully considered but they are not persuasive for the reasons below.

Regarding applicant's argument that "Nocker, et al. (2009) reference fails to teach elimination of amplification assay inhibitors by the addition of a chemical denaturant, and further (relevant to claim 6), Nocker, et al. (2009) does not teach performing PCR for correlation with viable cells for the diagnosis of septicemia", this argument is deemed to be NOT persuasive because applicants attack the references individually where, as here, the rejections citing Nocker et al. (2009) was based on teachings from Nocker et al. (2009) in combination with teachings and suggestions from other references (Nocker et al. (2007), Gebert et al. (2008), Horz et al. (2008) and McCann et al. (Apr 2014)).

In view of *In re Keller*, 208 USPQ 871 (CCPA 1981), it has been held that one cannot show non-obviousness by attacking references individually where, as here, the rejections are based on combinations of references.

In the combination of references that cites Nocker et al. (2009) of the Office action of 05/21/2014, neither Nocker et al. (2009) nor Nocker et al. (2007) were cited to teach the elimination of amplification assay inhibitors by the addition of a chemical denaturant.

Instead, Gerbert et al. as evidenced by Horz et al. and McCann et al., was cited to teach the

routine and conventional use of chemical denaturant in a MoLysis procedure which preceeds an amplification assay. Gelbert et al. particularly teach that PCR inhibitors are efficiently removed by MolYsis procedure (Gebert, pg 308, left col, 7th para and pg 313, right col, 2nd para). McCann et al. was cited as further teaching that the MolYsis procedure (pg 2, right col, section 2.4 and pg 3, left col, 1st para) is practiced using a MolYsis kit having a chaotropic buffer CM comprising a chemical denaturant/guanidinium hydrochloride, a strong chemical denaturant while Nocker et al. (2009) and/or Nocker et al. (2007) teach a qPCR amplification method for detecting the presence of a microbial DNA from only viable microbes (e.g. E. coli O157: H7, Listeria sp. (L. monocytogenes), Salmonella sp. (S. typhimurium) and Mycobacterium sp. (M. avium) of a mixture comprising both viable and dead cells).

In view of the combined teachings and suggestions of the reference cited above, it is within the purview of the ordinary skilled artisan seeking to perform the PCR amplification method for the detection of only DNA from viable microbes in a mixture that comprises both live and dead cells as taught by Nocker et al. (2009) or Nocker et al. (2007), to further optimize the PCR amplification assay in a manner as taught by Gerbert et al. who teach providing a chemical denaturant to pretreat a sample so as to eliminate amplification inhibitors that are present in the sample prior to performance of the amplification step. The rejection of record below provides a prima facie case of obviousness for how the prior art method of Gerbert et al. as evidenced by Horz et al. and McCann et al. improves the prior art methods of Nocker et al. (2009) and/or Nocker et al. (2007) thereby adding up to the instant invention.

9. Regarding applicant's argument (Remarks, pg 4, last para), that the various references which were cited and combined are unrelated and that these references do not suggest or provide one of ordinary skill in the art any guidance as to how they may/should be combined to produce applicant's claimed invention, or the resulting advantages of the invention, this argument is deemed unpersuasive because it has been held that the determination that a reference is from a nonanalogous art is twofold. First, we decide if the reference is within the field of the inventor's endeavor. If it is not, we proceed to determine whether the reference is reasonably pertinent to the particular problem with which the inventor was involved. *In re Wood*, 202 USPQ 171, 174.

In the instant case, as asserted above, it is within the purview of the ordinary skilled artisan to arrive at the instant invention because at least one of the cited prior art references teach the use of a chemical denaturant preceding an amplification reaction in order to remove PCR inhibitors and at least one of the cited prior art references teach an amplification assay wherein only DNA from viable cells are amplified and measured as specific signals indicative of the presence of viable microbes since the DNA from dead cells are excluded from amplification. Furthermore, the prior art references which teach such amplification assay teach/suggest measuring two or more time points and determining ratio of live to dead microbes.

The combination of the prior art elements above are *prima facie* obvious because one of ordinary skill in the art could have combined the elements and in the combination, each element merely would have performed the same function as it did separately (i.e., the use of a chemical denaturant prior to an amplification assay would have been recognized and/or applied as a known prior art "improvement" technique) and the results would have been predictable. The prior art of Gebert et al. as evidenced by Horz et al and McCann et al. provided a reason or the motivation to make an adaptation to the methods of Nocker et al. and there was a reasonable expectation of success at improvement of the method(s) of Nocker et al. by modifying the methods of Nocker et al. according to Gebert et al.

10. Regarding applicant's argument that the Examiner's conclusion of obviousness is based upon improper hindsight reasoning (pg 5, Remarks, 3rd para), this argument is deemed as unpersuasive because it is recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. *In re McLaughlin*, 443 F.2d 1392; 170 USPQ 209 (CCPA 1971).

11. Regarding applicant's argument that specification teach the instant method which is extensive and unpredictable (pg 4, Remarks, last para), this argument is deemed to be unpersuasive because there is no evidence other than Attorney argument to support the argument that the claimed method is unpredictable or extensive (see MPEP 2145).

Information Disclosure Statement

12. Applicant's submission of an information disclosure statement on 11/19/2014 is acknowledged. It is noted that the information disclosure statement of11/19/2014 fails to comply with the provisions of 37 CFR 1.97, 1.98 and MPEP § 609. It has been placed in the application file, but the information referred to therein has not been considered as to the merits. Applicant is advised that the date of any re-submission of any item of information contained in this information disclosure statement or the submission of any missing element(s) will be the date of submission for purposes of determining compliance with the requirements based on the time of filing the statement, including all certification requirements for statements under 37 CFR 1.97(e). See MPEP § 609.05(a).

The information disclosure statement of 11/19/2014 also fails to comply with 37 CFR 1.98(a)(2), which requires a legible copy of each cited foreign patent document; each non-patent literature publication or that portion which caused it to be listed; and all other information or that portion which caused it to be listed. It has been placed in the application file, but the information referred to therein has not been considered. Specifically, a complete copy of the following foreign patent documents does NOT appear to have been submitted: WO2007/1000762, WO2009/082747, WO2002/052034, WO2003/008636.

Claim Objections

13. Claim 2 is objected to because of the following informalities: Claim 2 contains numerous grammatical errors. Correction is required.

Claim Rejections - 35 USC § 112(b)

14. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

15. Claims 1-7 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph,

as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. Claims 2-7 are rejected solely for being dependent on claim 1. Claims 1 and 2 are vague and indefinite because it is unclear what order should the recited steps be performed in because the step of measuring time points of microbe-specific signal with an amplification assay is followed by the addition of chemical denaturant to eliminate amplification assay inhibitors. As recited, it is unclear how the artisan is to measure microbe specific signals with an amplification assay, prior to eliminating the inhibitors of an amplification assay by adding of a chemical denaturant.

As a matter of routine practice, it would generally follow that a chemical denaturant will be added prior to an amplification assay, then an amplification assay is performed followed by/simultaneous performed with the measuring of microbe specific signals and finally the ratio of live to dead microbes are determined from the result of the amplification assay.

However, the instant claim 1 does not recite this general/expected order. Clarification of the instant method steps is required as the current amendments to claim 1 do NOT clarify/make definite the order of the method steps of the instant claim 1 nor what the steps accomplish together as a whole.

Claims 3-7 are rejected as indefinite because they depend from claim 1.

16. Claim 6 is indefinite as it is unclear whether septicemia detection is required or whether the claim is reciting an inherent property of the method.

Claim Rejections - 35 USC § 112(d)

17. The following is a quotation of 35 U.S.C. 112(d):

> (d) REFERENCE IN DEPENDENT FORMS.—Subject to subsection (e), a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), fourth paragraph:

> Subject to the [fifth paragraph of 35 U.S.C. 112 (pre-AIA)], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

18. Claim 6 is rejected under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends.

Claim 6 recites the limitation "wherein performing the PCR assay provides in addition a correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia". It is unclear whether claim 6 requires septicemia detection or if an inherent capability or intended use of the method is recited. If the claim is simply reciting an intended use or inherent property, it is not further limiting.

Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements.

Claim Rejections - 35 USC § 103

19. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

20. This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

21. **Claims 1-7 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Nocker et al. (Mar 2009, J. Microbiol Methods, 76(3):253-61. Epub 2008 Dec 7: previously cited) in view of Nocker et al. (2007, J Microbiol. 70(2):252-60. Epub 2007 May 1: previously cited) and Gebert et al. (2008, J Infect. 57(4):307-16. Epub 2008 Aug 29: previously cited) as evidenced by Horz et al. (2008 Jan; 72(1):98-102) and McCann et al. (Apr 2014, J Microbiol Methods, 99:1-7. Epub 2014 Feb 3: previously cited).**

Regarding **claim 1**, Nocker et al. (2009) teach a method for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells or killed cells (entire document). The method of Nocker et al. comprises removing dead microbe cell DNA prior to obtaining a positive non-contaminated result from a nucleic acid amplification assay (by treating the mixture comprising intact and membrane compromised cells [pg 253, right col, 2nd para] with DNA intercalating dyes propidium monoazide PMA or ethidium monoazide EMA). Nocker et al. (2009) teach PMA and EMA is membrane-impermeant and selectively enters only the membrane-compromised cells and that upon exposure to strong visible light, EMA/PMA and dead cell DNA become crosslinked (pg 254, right col, section 2.5) and the crosslinked DNA cannot be amplified. Therefore, a nucleic acid amplification performed following a PMA or EMA treatment selectively indicates viable/intact cells are present in the mixture comprising intact and membrane compromised cells as PCR amplification of the dead cell DNA are inhibited (pg 253-254, Introduction).

Regarding **claims 1 and 4**, Nocker et al. (2009) teach measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes (as Nocker et al. teach qPCR amplification to detect Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella enterica serovar Typhimurium, Serratia marcescens and Escherichia coli O157:H7 (strain 932) [see pg 254-256, section 2.7-2.8 and 3.1 and pg 255, Tables 1-2] and particularly teach microbe specific amplification signal changes (pg 256, Fig. 1C). Nocker et al. teach microbe specific signal changes specific for Salmonella as a function of 8 different timepoints representing the different

proportions/cell numbers of viable Salmonella cells present in the mixture treated with PMA [pg, Figs. 3B and 3C].

Regarding **claim 1**, Nocker et al. (2009) teach determining the ratio of live to dead microbes present in the mixture (pg 254, left col, 2nd para and pg 256, right col, section 3.2 and pg 260, section 4.2).

Regarding **claim 5**, Nocker et al. (2009) teach a mixture comprising blood [pg 254, right col, section 2.5 and 2.6] to compare with a mixture that is an environmental sample.

Regarding **claim 7**, Nocker et al. (2009) teach signals from killed cells in the mixture are suppressed [pg 257, Fig. 2 see Mix VII] and membrane-compromised cells in the mixture are excluded from analysis (pg 258, Fig. 3 and pg 256, right col, section 3.2 and pg 259, left col, 1st and 2nd para and pg 260, left col, 2nd para).

Regarding **claims 1 and 3**, Nocker et al. (2009) do NOT teach eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant wherein the chemical denaturant comprises a mixture of one or more chemical agents.

Regarding **claim 6**, Nocker et al. (2009) did NOT teach performing the PCR assay provides correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia.

Regarding **claim 1**, Nocker et al. (2007) teach measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes since Nocker et al. (2007) teach quantitation and analysis of quantitative signal of the amplification assay for the microbes E. coli O157: H7, Listeria sp. (L. monocytogenes), Salmonella sp. (S. typhimurium) and Mycobacterium sp. (M. avium)[see pg 254-55, section 2.7 and pg 255, Fig. 1; pg 256, Fig. 2 and Fig. 3 and pg 257, Fig. 4] wherein Figs. 1A, 2A, 4A teach microbe-specific plate cell counts while Figs. 1D, 2D, 4B teach (ΔCt) or microbe-specific amplification-signal change cause by PMA treatment, wherein the ΔCt is negatively correlated with the number of viable microbes present.

Nocker et al. (2007) do NOT teach eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant.

Regarding **claims 1 and 3-5**, Gerbert et al. teach a pre-treatment of samples for amplification with a MolYsis procedure (Gebert, passim but particularly pg 313, right col, 2nd para) which involves lysis of human blood cells under chaotropic conditions while bacterial cells are unaffected, the released human DNA as well as any extracellular dead pathogenic DNA are then enzymatically degraded with a DNase (see Horz et al., pg 98, right col, 3rd para; pg 101, left col, 3rd para below Fig. 1), prior to the sedimentation of the viable bacterial cells, lysis of bacterial cell walls and extraction of bacterial DNA (pg 308, left col, 7th para) for further enrichment and detection by amplification (i.e. real-time PCR: Horz, pg 99, left col, 2nd para below table 1). Particularly regarding **claim 1,** Gerbert et al. teach PCR inhibitors are efficiently removed by MolYsis procedure (Gebert, pg 308, left col, 7th para and pg 313, right col, 2nd para).

Particularly regarding **claim 3,** McCann et al. teach MolYsis procedure (pg 2, right col, section 2.4 and pg 3, left col, 1st para) practiced using a MolYsis kit comprising reagents that includes a chaotrophic buffer CM (a chaotropic lysis buffer containing guanidinium hydrochloride), buffer DB1 (DNase buffer), MolDNaseB, buffer RS (normal saline), buffer RL, buffer RP (proteinase buffer), proteinase K, buffer CS, buffer AB (binding buffer), buffer WB, ethanol and a BugLysis reagent (pg 3, left col, 1st para). Particularly regarding **claim 1,** McCann et al. teach that buffer CM (comprising **denaturant**) is added to blood sample, incubated for 5 min after other buffers of the kit comprising one or more chemical agents are used (i.e. **claim 3**).

Regarding **claim 6,** Gebert et al. teach MolYsis procedure is useful when combined with detection techniques e.g. Gram-diff PCR (Conclusion section of the abstract and pg 313, right col, 4th para and pg 315, left col, 2nd para) for rapid identification of the causative agents of bloodstream infections (pg 308, left col, last para) and for detection of pathogenic fungi and bacteria for the diagnosis of sepsis (abstract, pg 308, left col, last para, pg 313, left col, Discussion and pg 315, left col, 2nd para).

It would have been obvious to a person of ordinary skill in the art wanting to selectively exclude DNA of dead cells from molecular detection of a mixture containing live and dead cells at the time of the invention, to combine the methods of Nocker et al. (2009) and Gebert et al. as evidenced by Horz and McCann so as to detect only signals from the viable microbes/cells of the mixture with a reasonable expectation of success. The motivation to combine the methods of Nocker et al. (2009) and the method of Gebert et al. comes from Nocker et al. (2009) who teach that bacterial diagnostics is hampered by inability to distinguish signals originating from live and dead cells (abstract) and Gebert et al. who teach detection of DNA of both viable and non-viable cells give false-positive PCR results (pg 314, right col, 1st para below Table 4). To combine the methods of Nocker et al. (2009) and Gebert et al., the ordinary skilled artisan would have been motivated to concentrate the cells of the mixture comprising viable, dead, killed and/or membrane compromised cells, perform a MolYsis procedure (i.e. the addition of CM followed by buffer DB1 and MolDNase B) to remove any contaminating human DNA and to degrade any extracellular naked DNA present in the mixture, centrifuge the mixture of cells and MolDNase B, CM buffer, DB1 buffer and discard supernatant, perform a PMA treatment on the recovered viable and membrane-compromised cells of the mixture comprising viable and membrane compromised cells so as to crosslink the DNA of the membrane-compromised cells with PMA, perform a DNA extraction with a MolYsis extraction procedure using remaining MolYsis buffers RL, Buglysis, buffer RP (proteinase buffer), proteinase K, buffer CS, buffer AB (binding buffer), buffer WB, ethanol etc. and perform a PCR reaction following the DNA extraction wherein the amplification of DNA of the membrane-compromised cells are inhibited from amplification as they are crosslinked.

One of ordinary skill in the art would have readily recognized that the combination of the methods of Nocker et al. (2009) and Gebert et al. would have provided positive non-contaminated result from viable cells excluding results from membrane compromised cells as the cited prior art each independently teach pre-detection sample treatment methods that prior to a nucleic acid amplification assay serves to selectively enrich DNA of viable cells for amplification. The combination of both methods as noted herein also selectively enriches the DNA of viable cells for amplification.

One of ordinary skill in the art would have been further motivated to provide the combined

methods for diagnosis of septicemia with a reasonable expectation of success as Gebert et al. teach suitability of the MolYsis procedure with other detection techniques (e.g. PCR) for rapid and accurate identification of pathogens causative of septicemia and teach microbes of septicemia (pg 310-314, Tables 1-4) for diagnosis of septicemia and as Nocker et al (2007) and Nocker et al. (2009) teach that the microbe-specific reduction in amplification signals of the nucleic acid amplification that follows a PMA treatment accurately corresponds to the live-dead ratio or the viable cells that are present in the mixture after a PMA treatment.

In view of the combined teachings and suggestions of all of the cited prior art references, the instant **claims 1 and 3-7** are prima facie obvious.

Regarding **claim 2**, Nocker et al. (2009) teach determining the ratio of live to dead microbes present in the mixture but did NOT explicitly teach ratio can be used as a measure of the effectiveness of a therapy or the efficacy of a treatment.

Regarding **claim 2**, Gebert et al. teach rapid detection of pathogens in blood from septic patients is essential for adequate antimicrobial therapy (abstract) but did NOT specifically teach determining the ratio of live to dead microbes present in the mixture as a measure of the effectiveness of a therapy or efficacy of a treatment.

Regarding **claim 2**, Nocker et al. teach (2007) teach monitoring disinfection efficacy by analyzing the quantitative signal of the amplification assay of the microbes E. coli O157: H7, L. monocytogenes, S. typhimurium and M. avium as a function of the concentration of hypochlorite quantity in ppm or concentration of benzalkonium quantity in ppm or UV exposure (min) or heat treatment [see pg 255, Fig. 1; pg 256, Fig. 2 and Fig. 3 and pg 257, Fig. 4 for specifics] but did not explicitly teach determining of the ratio of live to dead microbes per timepoint.

It would have been obvious to a person of ordinary skill in the art at the time of the invention to provide the ratio of live to dead microbes present in the mixture as a measure of the efficacy of a disinfection treatment since Nocker et al. (2009) teach that the signal intensities of an amplification following a PMA treatment, strongly reflects the ratio of live to dead microbe cells (pg 260, section 4.2) and Nocker et al. (2007) teach a linear correlation between loss of culturability and qPCR signal reduction for the range in disinfection strengths up to the point where colony counts dropped to zero (pg 259, left col, last para). In view of Nocker et al. (2009 and 2007), the combined methods of Nocker et al. (2009) and Gebert et al. as evidenced by Horz et al. and McCann et al. and Nocker et al. (2007) would have yielded qPCR signal intensities corresponding to the ratio of live to dead microbe cells, at each concentration of disinfectant thereby implicating the ratio of live to dead microbes present in the mixture at each concentration of disinfectant as the measure of the efficacy of disinfection with hypochlorite or benzalkonium.

In view of the combined teachings and suggestions of all of the cited prior art references, the instant **claim 2** is prima facie obvious.

Conclusion

22. No claims are free of the prior art. Applicant's amendment necessitated the new ground(s) of rejection presented in this office action. Accordingly, **THIS ACTION IS MADE FINAL**. See M.P.E.P § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO months of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Correspondence

23. Any inquiry concerning this communication or earlier communications from the examiner should be directed to OLAYINKA OYEYEMI whose telephone number is (571)270-5956. The examiner can normally be reached on M -Thurs 9-3 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/OLAYINKA OYEYEMI/
Examiner, Art Unit 1637

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

PTO/SB/08a (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	13/977,719
Filing Date	February 27, 2014
First Named Inventor	O'Hara, Shawn Mark
Art Unit	1637
Examiner Name	Oyeyemi
Attorney Docket Number	ZEUS 34 US

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]–Number[4]–Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	1	WO 2007/100762 A2	09-07-2007	Nocker, Andreas	P 3, 1. 9-24 Fig 3,4	
	2	WO 2009/082747 A1	07-02-2009	Zeus Scientific, Inc.	Abs., P 8,1. 6-23	
	3	WO 02/052034 A1	07-04-2002	Oshima, Joji	Abstract	
	4	WO 03/008636 A2	01-30-2003	Infectious Diagnostic Inc	Abstract	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13977719	O'HARA, SHAWN MARK
	Examiner	Art Unit
	OLAYINKA OYEYEMI	1637

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner
435	6.12	12/19/2014	OAO

SEARCH NOTES		
Search Notes	Date	Examiner
Updated PALM Inventor search	12/19/2014	OAO
Updated EAST Inventor and prior art search	12/19/2014	OAO

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	126	((Shawn) near2 (O''Hara)).INV.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2014/12/19 21:53
L2	2	"6210881".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2014/12/19 21:53
L3	0	l1 and detect near7 (dna near3 (dead or "non-viable"))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2014/12/19 21:55
L4	1	l1 and detect and (dna near7 (dead or "non-viable"))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2014/12/19 21:55

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
O'Hara, Shawn Mark

Docket No.: ZEUS 34 US

Application No. 13/977,719

Confirmation No.: 9440

Filed: February 27, 2014

Art Unit: 1637

Examiner: Oyeyemi

Title: **IMPROVED METHODS FOR**

DETERMINING CELL VIABILITY

USING MOLECULAR NUCLEIC ACID-BASED TECHNIQUES

RESPONSE AND AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is in response to the non-final Office Action mailed May 21, 2014 in the above-captioned application, and setting an initial three-month shortened period for response. Applicant is hereby responding within the six-month statutory period for response, and submits herewith the appropriate extension of the shortened period.

Responsive to the present Office Action, please amend the claims as set forth herein. Amendments to the claims begin on page 2 of this document.

Applicant's remarks/arguments in response to the Office Action begin on page 4 of this document.

Dated: *November 21, 2014*

Respectfully submitted,

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1767 Route 313
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/James L. Wilcox/

James L. Wilcox
Reg. No. 30,234
Attorney for Applicant

Amendments to the claims

1. (currently amended) A method for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells from a nucleic acid amplification assay thereby indicating that viable cells are present, ~~which~~ the method ~~comprises~~ comprising removing dead microbe cell DNA prior to obtaining a positive non contaminated result ~~from a nucleic acid amplification assay thereby indicating that viable cells are present,~~ by performing the steps of measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes, eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant, and determining the ratio of live to dead microbes present in the mixture.

2. (currently amended) In a ~~A~~ method ~~of claim 1, wherein~~ for the determination of the ratio of live to dead microbes present in ~~the~~ a mixture containing live and dead cells that can be used as a measure of the effectiveness of a therapy or the efficacy of a treatment by performing a nucleic acid amplification assay thereby indicating that viable cells are present, the improvement comprising measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes, eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant, and determining the ratio of live to dead microbes present in the mixture.

3. (original) The method of claim 1, wherein the chemical denaturant comprises a mixture of one or more chemical agents.

4. (original) The method of claim 1, wherein the amplification assay is a PCR assay.

5. (original) The method of claim 1, wherein the mixture comprises blood and other body fluids.

6. (currently amended) The method of claim 4, wherein performing the PCR assay provides in addition a correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia.

7. (original) The method of claim 1, wherein signals from killed cells in the mixture are suppressed and membrane-compromised cells in the mixture are excluded from analysis.

Remarks/Arguments

Claims 1-7 are pending in the present application. In the present Office Action, claim 1 was objected to by the examiner for the use of the word "which" in the preamble thereof. Claim 1 has been amended herein, in order to overcome the examiner's objection, by the use of the words "the method comprising" in place of the previously used phrase "which method comprises." Withdrawal of the objection to this claim, as amended, is now respectfully submitted to be proper and is accordingly requested.

Claims 1-7 stand rejected in the present Office Action under 35 USC section 112 on various grounds, with the examiner pointing out very specific reasons for grounds of rejection with respect to claims 2 and 6. In order to directly address these rejections, claims 1, 2 and 6 have been amended herein. In particular, claim 1 has been amended to specifically recite the order of steps of the claimed method, claim 2 has been re-written in independent form pursuant to the examiner's kind suggestion and to particularly point out and distinctly claim the improvement provided by the invention, and claim 6 has been amended in order to particularly point out the additional feature of the claimed invention for use in the diagnosis of septicemia, as recited therein.

In view of the above claim amendments, it is respectfully urged that the present section 112 rejections with respect to the original claims have been fully overcome and should be withdrawn. No new matter has been introduced by the amendments herein.

In the present Office Action, section 13, the examiner has additionally rejected claims 1 through 7 under pre-AIA 35 U.S.C. section 103(a) over a combination of literature references of record: two Nocker, et al. references (2007 and 2009) and the Gebert, et al. reference, as evidenced by the Horz, et al. and McCann, et al. references. Applicant traverses the examiner's grounds for this rejection in view of the amendment of the claims herein and the arguments below, and respectfully requests that such rejection be withdrawn

The examiner advances various rationales for combining the references cited in the instant Office Action in various ways to deem Applicant's claimed invention as obvious to one skilled in the art at the time the invention was made. However, it is respectfully pointed out that in order for a valid combination of various references, which are inherently not themselves related in any way, to render a claimed invention obvious, the references themselves must actually suggest or provide one skilled in the art some guidance as to how they may be combined. In this case, none of these references teach, or so much as suggest, any combination in any way that would render Applicant's invention obvious. Absent any such teaching or suggestion, there is nothing that can serve as a guide or motivation for one skilled in the art to combine them to produce Applicant's claimed invention, and the resulting advantages of the invention. At best, one skilled in the art, in order to make the claimed invention from the piecemeal disclosures of these references, would have to conduct extensive and unpredictable experiments such as those described in Applicant's Specification, in order to achieve the presently claimed invention and to realize the results thereof.

In fact, the examiner in the instant Office Action acknowledges that the Nocker, et al. (2009) reference fails to teach elimination of amplification assay inhibitors by the addition of a chemical denaturant, and further (relevant to claim 6) does not teach performing PCR for correlation with viable cells for the diagnosis of septicemia. The examiner also acknowledges that the Nocker, et al. (2007) fails to teach elimination of amplification assay inhibitors by the addition of a chemical denaturant.

It is again respectfully asserted that in the absence of such teachings, or even suggestions thereof, in the cited references themselves, there is no valid combination of these references available to provide a grounds of rejection of Applicant's claims. In particular, and even more specifically-- none of the references alone, or in any combination, fairly teach or suggest the elimination of amplification assay inhibitors by the addition of a chemical denaturant. This is a specific recited element of Applicant's independent claims of the present application, and of the dependent claims following therefrom.

Accordingly, it is urged that only by combining the isolated disclosures of the cited references with knowledge, in hindsight, of the teachings of Applicant's own disclosure in the instant application, could one skilled in the art be led to achieving the development of the presently claimed invention. It is well settled that a rejection of claims based on the above premise – using improper hindsight reconstruction -- cannot be maintained, and therefore it is respectfully requested that the outstanding rejection of the claims under 35 USC section 103 must properly be withdrawn.

In summary, Applicant's invention as presently claimed is in no way disclosed or suggested by the references of record, or by any combination thereof. Only by the application of hindsight reconstruction using Applicant's own disclosure, which would be improper under these circumstances, could Applicant's presently claimed invention be in any way considered obvious, and therefore un-patentable, in view of these references.

In view of the amendments herein and the foregoing remarks, it is respectfully urged that all of the grounds asserted in the present Office Action for the rejection of claims 1-7 have been overcome, and that accordingly such rejection should be withdrawn and these claims passed to allowance. Such favorable action is respectfully requested.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/977,719	02/27/2014	Shawn Mark O'Hara	ZEUS 34 US	9440

74500 7590 05/21/2014

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EXAMINER	
OYEYEMI, OLAYINKA A	

ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
05/21/2014	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 13/977,719	Applicant(s) O'HARA, SHAWN MARK	
	Examiner OLAYINKA OYEYEMI	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-7* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-7* is/are rejected.

8)☒ Claim(s) *1* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *06/30/2013* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Priority

2. This application is a 371 of PCT/US11/67329 filed on December 27, 2011 and claims the benefit of U.S. Serial Application No. 61/428,892 filed on **December 31, 2010**.

Status of the claims

3. Claims 1-7 are pending.

Claim Objections

4. Claim 1 is objected to because of the following informalities: Claim 1 recites the preamble "A method for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells, which method comprises". The word "which" makes the preamble grammatically incorrect. Appropriate correction is required.

Claim Rejections - 35 USC § 112(b)

5. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 1-7 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. Claims 2-7 are rejected solely for being dependent on claim 1.

7. Claim 1 is confusing because it is unclear what order should the recited steps be performed in as

the step of measuring time points of microbe-specific signal is followed by the addition of chemical denaturant to eliminate amplification assay inhibitors. Claims 2-7 are also indefinite since they depend from claim 1.

8. Claim 2 is confusing because it is unclear whether the claim is simply stating an inherent property of the method, i.e., that it may be used to monitor treatment with an anti-microbial agent, or whether the claim requires treatment with an anti-microbial agent and determining the ratio of live to dead microbes.

9. Claim 6 is indefinite as it is unclear whether septicemia detection is required or whether the claim is reciting an inherent property of the method.

Claim Rejections - 35 USC § 112(d)

10. The following is a quotation of 35 U.S.C. 112(d):

> (d) REFERENCE IN DEPENDENT FORMS.—Subject to subsection (e), a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), fourth paragraph:

> Subject to the [fifth paragraph of 35 U.S.C. 112 (pre-AIA)], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

11. Claims 2 and 6 are rejected under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends.

12. Claim 2 recites the limitation "wherein the determination of the ratio of live to dead microbes present in the mixture can be used as a measure of the effectiveness of a therapy or the efficacy of a treatment". As noted above, the requirements of the claim are unclear. If the claim is simply stating an intended use i.e. an association of the ratio with therapy/treatment, it does not further limit the value of determined ratio of claim 1.

13. Likewise, as discussed above, it is unclear whether claim 6 requires septicemia detection or if an

inherent capability or intended use of the method is recited. If the claim is simply reciting an intended use or inherent property, it is not further limiting.

14. Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements.

Claim Rejections - 35 USC § 103

11. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

12. This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

13. **Claims 1-7 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Nocker et al. (Mar 2009, J. Microbiol Methods, 76(3):253-61. Epub 2008 Dec 7) in view of Nocker et al. (2007, J Microbiol. 70(2):252-60. Epub 2007 May 1) and Gebert et al. (2008, J Infect. 57(4):307-16. Epub 2008 Aug 29) as evidenced by Horz et al. (2008 Jan; 72(1):98-102) and McCann et al. (Apr 2014, J Microbiol Methods, 99:1-7. Epub 2014 Feb 3).**

Regarding **claim 1**, Nocker et al. (2009) teach a method for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells or killed cells (entire document). The method of Nocker et al. comprises removing dead microbe cell DNA prior to obtaining a positive non-contaminated result from a nucleic acid amplification assay (by treating the mixture comprising intact and membrane compromised cells [pg 253, right col, 2nd para] with DNA intercalating dyes propidium monoazide PMA or ethidium monoazide EMA). Nocker et al. (2009) teach PMA and EMA is membrane-impermeant and selectively enters only the membrane-compromised cells and that upon exposure to strong visible light, EMA/PMA and dead cell DNA become crosslinked (pg 254, right col, section 2.5) and the crosslinked DNA cannot be amplified. Therefore, a nucleic acid amplification performed following a PMA or EMA treatment selectively indicates viable/intact cells are present in the mixture comprising intact and membrane compromised cells as PCR amplification of the dead cell DNA are inhibited (pg 253-254, Introduction).

Regarding **claims 1 and 4**, Nocker et al. (2009) teach measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes (as Nocker et al. teach qPCR amplification to detect Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella enterica serovar Typhimurium, Serratia marcescens and Escherichia coli O157:H7 (strain 932) [see pg 254-256, section 2.7-2.8 and 3.1 and pg 255, Tables 1-2] and particularly teach microbe specific amplification signal changes (pg 256, Fig. 1C). Nocker et al. teach microbe specific signal changes specific for Salmonella as a function of 8 different timepoints representing the different proportions/cell numbers of viable Salmonella cells present in the mixture treated with PMA [pg, Figs. 3B and 3C].

Regarding **claim 1**, Nocker et al. (2009) teach determining the ratio of live to dead microbes present in the mixture (pg 254, left col, 2nd para and pg 256, right col, section 3.2 and pg 260, section 4.2).

Regarding **claim 5**, Nocker et al. (2009) teach a mixture comprising blood [pg 254, right col, section 2.5 and 2.6] to compare with a mixture that is an environmental sample.

Regarding **claim 7**, Nocker et al. (2009) teach signals from killed cells in the mixture are

suppressed [pg 257, Fig. 2 see Mix VII] and membrane-compromised cells in the mixture are excluded from analysis (pg 258, Fig. 3 and pg 256, right col, section 3.2 and pg 259, left col, 1st and 2nd para and pg 260, left col, 2nd para).

Regarding **claims 1 and 3**, Nocker et al. (2009) do NOT teach eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant wherein the chemical denaturant comprises a mixture of one or more chemical agents.

Regarding **claim 6**, Nocker et al. (2009) did NOT teach performing the PCR assay provides correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia.

Regarding **claim 1**, Nocker et al. (2007) teach measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes since Nocker et al. (2007) teach quantitation and analysis of quantitative signal of the amplification assay for the microbes E. coli O157: H7, Listeria sp. (L. monocytogenes), Salmonella sp. (S. typhimurium) and Mycobacterium sp. (M. avium)[see pg 254-55, section 2.7 and pg 255, Fig. 1; pg 256, Fig. 2 and Fig. 3 and pg 257, Fig. 4] wherein Figs. 1A, 2A, 4A teach microbe-specific plate cell counts while Figs. 1D, 2D, 4B teach (ΔCt) or microbe-specific amplification-signal change cause by PMA treatment, wherein the ΔCt is negatively correlated with the number of viable microbes present.

Nocker et al. (2007) do NOT teach eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant.

Regarding **claims 1 and 3-5**, Gerbert et al. teach a pre-treatment of samples for amplification with a MolYsis procedure (Gebert, passim but particularly pg 313, right col, 2nd para) which involves lysis of human blood cells under chaotropic conditions while bacterial cells are unaffected, the released human DNA as well as any extracellular dead pathogenic DNA are then enzymatically degraded with a DNase

(see Horz et al., pg 98, right col, 3rd para; pg 101, left col, 3rd para below Fig. 1), prior to the sedimentation of the viable bacterial cells, lysis of bacterial cell walls and extraction of bacterial DNA (pg 308, left col, 7th para) for further enrichment and detection by amplification (i.e. real-time PCR: Horz, pg 99, left col, 2nd para below table 1). Particularly regarding **claim 1,** Gerbert et al. teach PCR inhibitors are efficiently removed by MolYsis procedure (Gebert, pg 308, left col, 7th para and pg 313, right col, 2nd para).

Particularly regarding **claim 3,** McCann et al. teach MolYsis procedure (pg 2, right col, section 2.4 and pg 3, left col, 1st para) practiced using a MolYsis kit comprising reagents that includes a Chaotrophic buffer CM (a chaotropic lysis buffer containing guanidinium hydrochloride), buffer DB1 (DNase buffer), MolDNaseB, buffer RS (normal saline), buffer RL, buffer RP (proteinase buffer), proteinase K, buffer CS, buffer AB (binding buffer), buffer WB, ethanol and a BugLysis reagent (pg 3, left col, 1st para). Particularly regarding **claim 1,** McCann et al. teach that buffer CM (comprising **denaturant**) is added to blood sample, incubated for 5 min after other buffers of the kit comprising one or more chemical agents are used (i.e. **claim 3**).

Regarding **claim 6,** Gebert et al. teach MolYsis procedure is useful when combined with detection techniques e.g. Gram-diff PCR (Conclusion section of the abstract and pg 313, right col, 4th para and pg 315, left col, 2nd para) for rapid identification of the causative agents of bloodstream infections (pg 308, left col, last para) and for detection of pathogenic fungi and bacteria for the diagnosis of sepsis (abstract, pg 308, left col, last para, pg 313, left col, Discussion and pg 315, left col, 2nd para).

It would have been obvious to a person of ordinary skill in the art wanting to selectively exclude DNA of dead cells from molecular detection of a mixture containing live and dead cells at the time of the invention, to combine the methods of Nocker et al. (2009) and Gebert et al. as evidenced by Horz and McCann so as to detect only signals from the viable microbes/cells of the mixture with a reasonable expectation of success. The motivation to combine the methods of Nocker et al. (2009) and the method of Gebert et al. comes from Nocker et al. (2009) who teach that bacterial diagnostics is hampered by inability to distinguish signals originating from live and dead cells (abstract) and Gebert et al. who teach detection of DNA of both viable and non-viable cells give false-positive PCR results (pg 314, right col, 1st

para below Table 4). To combine the methods of Nocker et al. (2009) and Gebert et al., the ordinary skill artisan would have been motivated to concentrate the cells of the mixture comprising viable, dead, killed and/or membrane compromised cells, perform a MolYsis procedure (i.e. the addition of CM followed by buffer DB1 and MolDNase B) to remove any contaminating human DNA and to degrade any extracellular naked DNA present in the mixture, centrifuge the mixture of cells and MolDNase B, CM buffer, DB1 buffer and discard supernatant, perform a PMA treatment on the recovered viable and membrane-compromised cells of the mixture comprising viable and membrane compromised cells so as to crosslink the DNA of the membrane-compromised cells with PMA, perform a DNA extraction with a MolYsis extraction procedure using remaining MolYsis buffers RL, Buglysis, buffer RP (proteinase buffer), proteinase K, buffer CS, buffer AB (binding buffer), buffer WB, ethanol etc. and perform a PCR reaction following the DNA extraction wherein the amplification of DNA of the membrane-compromised cells are inhibited from amplification as they are crosslinked.

One of ordinary skill in the art would have readily recognized that the combination of the methods of Nocker et al. (2009) and Gebert et al. would have provided positive non-contaminated result from viable cells excluding results from membrane compromised cells as the cited prior art each independently teach pre-detection sample treatment methods that prior to a nucleic acid amplification assay serves to selectively enrich DNA of viable cells for amplification. The combination of both methods as noted herein also selectively enriches the DNA of viable cells for amplification.

One of ordinary skill in the art would have been further motivated to provide the combined methods for diagnosis of septicemia with a reasonable expectation of success as Gebert et al. teach suitability of the MolYsis procedure with other detection techniques (e.g. PCR) for rapid and accurate identification of pathogens causative of septicemia and teach microbes of septicemia (pg 310-314, Tables 1-4) for diagnosis of septicemia and as Nocker et al (2007) and Nocker et al. (2009) teach that the microbe-specific reduction in amplification signals of the nucleic acid amplification that follows a PMA treatment accurately corresponds to the live-dead ratio or the viable cells that are present in the mixture after a PMA treatment.

In view of the combined teachings and suggestions of all of the cited prior art references, the

instant **claims 1 and 3-7** are prima facie obvious.

Regarding **claim 2**, Nocker et al. (2009) teach determining the ratio of live to dead microbes present in the mixture but did NOT explicitly teach ratio can be used as a measure of the effectiveness of a therapy or the efficacy of a treatment.

Regarding **claim 2**, Gebert et al. teach rapid detection of pathogens in blood from septic patients is essential for adequate antimicrobial therapy (abstract) but did NOT specifically teach determining the ratio of live to dead microbes present in the mixture as a measure of the effectiveness of a therapy or efficacy of a treatment.

Regarding **claim 2**, Nocker et al. teach (2007) teach monitoring disinfection efficacy by analyzing the quantitative signal of the amplification assay of the microbes E. coli O157: H7, L. monocytogenes, S. typhimurium and M. avium as a function of the concentration of hypochlorite quantity in ppm or concentration of benzalkonium quantity in ppm or UV exposure (min) or heat treatment [see pg 255, Fig. 1; pg 256, Fig. 2 and Fig. 3 and pg 257, Fig. 4 for specifics] but did not explicitly teach determining of the ratio of live to dead microbes per timepoint.

It would have been obvious to a person of ordinary skill in the art at the time of the invention to provide the ratio of live to dead microbes present in the mixture as a measure of the efficacy of a disinfection treatment since Nocker et al. (2009) teach that the signal intensities of an amplification following a PMA treatment, strongly reflects the ratio of live to dead microbe cells (pg 260, section 4.2) and Nocker et al. (2007) teach a linear correlation between loss of culturability and qPCR signal reduction for the range in disinfection strengths up to the point where colony counts dropped to zero (pg 259, left col, last para). In view of Nocker et al. (2009 and 2007), the combined methods of Nocker et al. (2009) and Gebert et al. as evidenced by Horz et al. and McCann et al. and Nocker et al. (2007) would have

yielded qPCR signal intensities corresponding to the ratio of live to dead microbe cells, at each concentration of disinfectant thereby implicating the ratio of live to dead microbes present in the mixture at each concentration of disinfectant as the measure of the efficacy of disinfection with hypochlorite or benzalkonium.

In view of the combined teachings and suggestions of all of the cited prior art references, the instant **claim 2** is prima facie obvious.

Conclusion

14. No claims are free of the prior art. The art made of record and not relied upon is considered pertinent to applicant's disclosure. Hansen et al. (Epub 2009 Jun 17, J Clin Microbiol. 2009, 47(8):2629-31), Vesper et al. (Feb. 2008, J Microbiol Methods, 72(2):180-4. Epub 2007 Nov 28), MolYsis manual (Jan 2012).

Correspondence

15. Any inquiry concerning this communication or earlier communications from the examiner should be directed to OLAYINKA OYEYEMI whose telephone number is (571)270-5956. The examiner can normally be reached on M -Thurs 9-3 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-

1000.

/OLAYINKA OYEYEMI/
Examiner, Art Unit 1637

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 13/977,719	Applicant(s)/Patent Under Reexamination O'HARA, SHAWN MARK	
	Examiner OLAYINKA OYEYEMI	Art Unit 1637	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Gebert S, Siegel D, Wellinghausen N. Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis. J Infect. 2008 Oct; 57(4):307-16. Epub 2008 Aug 29.
	V	Hansen WL, Bruggeman CA, Wolffs PF. Evaluation of new preanalysis sample treatment tools and DNA isolation protocols to improve bacterial pathogen detection in whole blood. J Clin Microbiol. 2009 Aug; 47(8):2629-31. Epub 2009 Jun 17.
	W	Nocker A, Mazza A, Masson L, Camper AK, Brousseau R. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J. Microbiol Methods. 2009 Mar; 76(3):253-61. Epub 2008 Dec 7.
	X	Nocker A, Sossa KE, Camper AK. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J Microbiol Methods. 2007 Aug; 70(2):252-60. Epub 2007 May 1.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 13/977,719	Applicant(s)/Patent Under Reexamination O'HARA, SHAWN MARK	
	Examiner OLAYINKA OYEYEMI	Art Unit 1637	Page 2 of 2

U.S. PATENT DOCUMENTS

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	A	US-			
	B	US-			
	C	US-			
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FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Horz HP, Scheer S, Huenger F, Vianna ME, Conrads G. Selective isolation of bacterial DNA from human clinical specimens. J Microbiol Methods. 2008 Jan; 72(1):98-102. Epub 2007 Nov 28.
	V	MolYsis manual, Jan 2012. Reseach in Molecular Microbiol No. submitted by Marina Linow, Molzym GmBH & Co. KG 28359 Bremen, Germany. pg 1-2
	W	McCann CD, Jordan JA. Evaluation of MolYsis™ Complete5 DNA extraction method for detecting Staphylococcus aureus DNA from whole blood in a sepsis model using PCR/pyrosequencing. J Microbiol Methods. 2014 Apr; 99:1-7. Epub 2014 Feb 3.
	X	Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S, Vesper A. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J Microbiol Methods. 2008 Feb;72(2):180-4. Epub 2007 Nov 28.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13977719	O'HARA, SHAWN MARK
	Examiner	Art Unit
	OLAYINKA OYEYEMI	1637

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner
435	6.12	05/01/2014	OAO

SEARCH NOTES		
Search Notes	Date	Examiner
PALM Inventor search	05/01/2014	OAO
EAST Inventor search	05/01/2014	OAO
International Preliminary Report on patentability Chapter I (PCT/US2011/067329) submitted July 2, 2013	05/01/2014	OAO
Google scholar NPL search	05/01/2014-05/12/2014	OAO

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	30	((Shawn) near2 (O''Hara)).INV.	US-PGPUB; USPAT; USOCR	OR	ON	2014/05/01 15:56
S2	116	((Shawn) near2 (O''Hara)).INV.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2014/05/01 15:57
S3	1	"6210881".pn.	US-PGPUB; USPAT; USOCR	OR	ON	2014/05/08 19:50

What is claimed is:

1. A method for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells, which method comprises removing dead microbe cell DNA prior to obtaining a positive non contaminated result from a nucleic acid amplification assay thereby indicating that viable cells are present, measuring two or more time points of microbe-specific signal increases from the amplification assay as an indication of the presence of viable microbes, eliminating amplification assay inhibitors from the mixture by the addition of a chemical denaturant, and determining the ratio of live to dead microbes present in the mixture.

2. The method of claim 1, wherein the determination of the ratio of live to dead microbes present in the mixture can be used as a measure of the effectiveness of a therapy or the efficacy of a treatment.

3. The method of claim 1, wherein the chemical denaturant comprises a mixture of one or more chemical agents.

4. The method of claim 1, wherein the amplification assay is a PCR assay.

5. The method of claim 1, wherein the mixture comprises blood and other body fluids.

6. The method of claim 4, wherein performing the PCR assay provides correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia.

7. The method of claim 1, wherein signals from killed cells in the mixture are suppressed and membrane-compromised cells in the mixture are excluded from analysis.

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IMPROVED METHODS FOR DETERMINING CELL VIABILITY USING MOLECULAR NUCLEIC ACID-BASED TECHNIQUES

CROSS REFERENCE TO RELATED APPLICATION

This application is a non-provisional application, which is incorporated by reference herein and claims priority of US Provisional Application No. 61/428,892, filed December 31, 2010.

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to methods for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells, and in particular relates to improved methods for performing direct Polymerase Chain Reaction (PCR) techniques in blood and other body fluids for correlation with viable microbe cells from Bacteremia, Fungemia, Viremia and other types of parasite containing samples. The improved methods provided by the invention are particularly advantageous for the diagnosis of septicemia.

BACKGROUND ART

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude other additives, components, integers or steps.

In diagnosing septicemia the time to result (TTR) is the most important determination of patient survival. Currently, blood culture is the gold standard, but is relatively slow, generating viable microorganisms for subsequent identification with a approximate median time of 15 hours (in the general range of 3 hours to 5 days) to turn positive, after which microbe identification typically can add another 1-2 days for the analysis. Molecular methods such as PCR offer vastly improved TTR for microbe identification, but suffer from a lack of specificity primarily due to inadequate selectivity of viable microbe cells during sample preparation. Traditional septicemia PCR testing of blood conventionally requires costly DNA isolations to remove PCR inhibitors,

but isolation also causes false positives and loss of sensitivity compared to the gold standard of blood culture, primarily due to the inclusion of DNA from dead microbe cells and sample processing dependent losses during the DNA isolation procedure.

Traditionally, septicemia blood sample PCR preparations have always isolated DNA from blood and blood products to remove the long and well known blood derived PCR Inhibitors of Taq polymerases (see the Klouche and Schroder article cited below). Recently in an attempt to overcome this inhibition some groups have developed PCR-enhancing mixtures as well as modified thermal-stable polymerases (for example, the well-known "omni taq" and "Phusion" techniques) engineered to reduce the inhibitory affect of blood products on these polymerases (see JMD, 2010; 12(2), pp.152-161). However the constraints of both of these approaches still suffer from either a lack of sensitivity due to low tolerated blood volume, and the high costs and loss of sample and high complexity that are associated with isolation systems. Furthermore DNA Isolation systems often include the cell free DNA from dead cells, which can have the effect of causing confounding false positives.

Klouche, M. and Schroder, U. in an article entitled "Rapid methods for diagnosis of bloodstream infections," published in Clin. Chem. Lab. Med., 2008; 46(7), pp. 888-908, disclose that direct nucleic acid-based detection and identification of microbial pathogens in blood from patients can be a promising tool for rapid diagnosis of bloodstream infections. According to this article, the significance of detection of circulating bacterial or fungal nucleic acids by broad-range molecular approaches for routine workup of bloodstream infections, however, is at present not clear. Encouraging issues for improvement of quality and reproducibility of molecular

diagnostic applications in bloodstream infections include selective enrichment procedures for bacterial nucleic acids, blocking or elimination methods of excess human DNA, and use of viability markers to discriminate clinically relevant findings, as shown in experience from microbial safety analysis. Despite the currently expensive and technically demanding technologies, disease-oriented multiplex PCR, pathogen microarrays and proteomic profiling have the potential to evolve as important rapid and high-throughput diagnostic means for infectious disease diagnosis. At present, three main considerations preclude the unique application of molecular technologies in routine diagnosis of bloodstream infections: the difficulties in interpretation of the NAT results due to 1) the high risk of external contamination, the extended persistence of nucleic acids after infection, and transient bacteraemia, 2) the limited analytical sensitivity for clinically relevant low bacterial loads, and for detection of certain bacteria and fungi, and 3) the lack of routine antimicrobial susceptibility testing by molecular as well as by proteomic testing.

Differentiation of live and dead cells is an important challenge in microbial diagnostics. Metabolic and reproductive activity, and, in the case of pathogenic microorganisms, the potential health risk are limited to the live portion of a mixed microbial population. Four physiological states are used in the conventional art to distinguish, in flow cytometry using fluorescent stains: reproductively viable, metabolically active, intact and permeabilized cells. Depending on the conditions, all stages except the permeabilized cells can have the potential of recovery upon resuscitation and thus have to be considered potentially live. Due to the relatively long persistence of DNA after cell death in the range between days to 3 weeks, DNA-based diagnostics tend to overestimate the number of live cells. DNA extracted from a sample can

originate from cells in any of the four mentioned physiological states including the dead permeabilized cells. Detection of the latter, however, is not desired. The most important criterion for distinguishing between viable and irreversibly damaged cells is membrane integrity. Sorting out noise derived from membrane-compromised cells helps to assign metabolic activities and health risks to the intact and viable portion of bacterial communities. Live cells with intact membranes have been distinguished by their ability to exclude DNA-binding dyes that easily penetrate dead or membrane-compromised cells.

Recently, EMA-PCR was reported to be an easy-to-use alternative to microscopic or flow-cytometric analyses to distinguish between live and dead cells. This diagnostic DNA-based method combines the use of a live-dead discriminating dye with the speed and sensitivity of real-time PCR. Ethidium monoazide (EMA). a DNA-intercalating dye with the azide group allowing covalent binding of the chemical to DNA upon exposure to bright visible light (maximum absorbance at 460 nm), has been used in this regard. Cells are exposed to EMA for 5 minutes allowing the dye to penetrate dead cells with compromised cell walls/membranes and to bind to their DNA. Photolysis of EMA using bright visible light produces a nitrene that can form a covalent link to DNA and other molecules.

Photo-induced cross-linking has been reported to inhibit PCR amplification of DNA from dead cells. It has been recently shown that EMA-crosslinking to DNA actually render the DNA insoluble, and leads to loss together with cell debris during genomic DNA extraction. Unbound EMA, which remains free in solution, can be simultaneously inactivated by reacting with water molecules. The resulting hydroxylamine is no longer capable of covalently binding to DNA. DNA from viable cells, protected from reactive EMA before light-exposure by an intact cell

membrane/cell wall, is therefore not affected by the inactivated EMA after cell lysis. Therefore, EMA treatment of bacterial cultures comprised of a mixture of viable and dead cells thus leads to selective removal of DNA from dead cells. The species tested were E. coli 0157:H7, Salmonella typhimuŕiim, Listeria monocytogenes and Campylobacter Jejuni. These studies did not examine, however, the selective loss of DNA from dead cells.

Though this technique is promising, the use of EMA prior to DNA extraction has been found to suffer from a major drawback. In some cases, the treatment also resulted in loss of approximately 60% of the genomic DNA of viable cells harvested in log phase. It has been observed that EMA also readily penetrates viable cells of other bacterial species resulting in partial DNA loss. This lack of selectivity and of overall applicability has led to testing of a newly developed alternative chemical: Propidium monoazide (PMA). In a published patent application, WO/2007/100762 to Nocker, et al., published September 7, 2007, there is disclosed the suitability of PMA to selectively remove detection of genomic DNA of dead cells from bacterial cultures with defined portions of live and dead cells. PMA is identical to propidium iodide (PI), except that the additional presence of an azide group allows crosslinkage to DNA upon light-exposure. PI has been extensively used to identify dead cells in mixed populations. The higher charge of the PMA molecule (2 positive charges compared to only one in the case of EMA) and because selective staining of nonviable cells with PI had been successfully performed on a wide variety of cell types, led those in the field to believe that the use of PMA might mitigate the drawbacks observed with EMA. In this published patent, PMA concentration and incubation time were optimized with one gram-negative and one gram-positive organism before applying these parameters to the study of a broad-spectrum of different bacterial species. The disclosed method purportedly limits molecular diagnostics to the portion of a microbial community with

intact cell membranes. This is achieved by exposing a mixture of intact and membrane-compromised cells to a phenanthridium derivative. In a disclosed preferred embodiment, PCR is performed using genomic DNA from the mixture as a template.

Also, Published U.S. Patent Application No. 2008/0160528, to Lorenz, published July 3, 2008, discloses the use of nucleases, especially DNA-degrading nucleases, for degrading nucleic acids in the presence of one or several chaotropic agents and/or one or several surfactants. This patent application further discloses a method for purifying RNA from mixtures of DNA and RNA as well as kits for carrying out such a method. Also disclosed is a method for specifically isolating nucleic acids from microbial cells provided in a mixed sample which additionally comprises higher eukaryotic cells as well as kits for carrying out such a method.

Another published patent application, WO/2001/077379 to Rudi, et al., published October 18, 2001, discloses methods of detecting cells in a sample and for obtaining quantitative information about cell populations within a sample. In particular, a method is disclosed for distinguishing between living and dead cells in a sample. The method comprises contacting the sample with a viability probe which modifies the nucleic acid of dead cells within the sample, and detecting nucleic acid from the cells in the sample. Also described is a method of detecting cells in a sample, the method comprising: (a) contacting the sample with a viability probe which labels the nucleic acid of dead cells within the sample; (b) separating the nucleic acid from the cells into labeled and non-labelled fractions; and (c) detecting the nucleic acid in one or both of the fractions.

SUMMARY OF THE INVENTION

In view of the foregoing background art, it can be seen that a paradigm shift would be to develop a method that effectively discriminates live vs. dead microbe cell DNA prior to molecular nucleic-acid based analysis techniques (for example before PCR set up), and that also circumvents the costly negative effects of traditional isolation designed to remove, e.g., PCR inhibitors and concentrate target DNA. Surprisingly, in accordance with the practice of an embodiment of the present invention, it has been shown that PCR correlates with viable microbe cells derived from blood, employing a combination of selective blood cell lysis, washing (and or) DNase along with subsequent microbe cell lysis and PCR.

Thus, in contrast to the conventional methods described above, the present invention seeks to realize the potential TTR advantage of molecular nucleic-acid based techniques, including PCR, by dramatically simplifying costly DNA isolations and sample preparation, and by not isolating DNA, but rather by performing a rapid and simple direct-analysis on crude microbe lysates after a rapid separation of the dead microbe DNA and cells, resulting in the selective enrichment of viable microbe cells. This is particularly and unexpectedly advantageous in the diagnosis of septicemia, and is accomplished according to a preferred embodiment of the present invention by:

I. The removal of confounding dead microbe cell DNA prior to a positive non contaminated PCR result indicates that viable cells are present, and as such the PCR result will indicate

the presence of viable septicemia microbe(s), i.e., blood microbe PCR = viable septicemia microbes.

II. As is well known, dead microbe cells from blood cannot grow in blood culture, thus any two or more time points measuring significant microbe-specific PCR signal increases from a single blood culture bottle must be measuring viable microbes.

III. PCR inhibitors from blood can be eliminated via a simple combination of chemical denaturants (chaotropes: detergents, pH, salts, organic chemical based differential salvation via dipole moment such as alcohols and amine containing compounds & enzymes such as nucleases, proteinases etc.) and washing, thereby circumventing DNA isolation and enabling microbe lysate-Direct-PCR.

IV. The ratio of live/dead microbes present in blood and blood culture can then be used as a measure of the effectiveness of a therapy and of testing the efficacy of treatment.

Accordingly, it is an objective of the present invention to provide improved methods for selectively excluding, from molecular detection, DNA of dead cells from a mixture containing live and dead cells.

It is a further objective of the of the present invention to provide improved methods that effectively discriminate live vs. dead microbe cell DNA prior to molecular nucleic-acid based analysis or PCR set up, and that also circumvents the costly negative effects of traditional isolation such as those designed to remove PCR inhibitors and concentrate target DNA.

It is another objective of the present invention to provide methods of correlating results of PCR and other molecular analysis techniques with the presence of viable microbe cells derived from blood, for example by employing a combination of selective blood cell lysis, washing (and or) DNase along with subsequent microbe cell lysis and PCR.

It is yet another objective of the present invention to provide improved methods for performing direct PCR techniques in blood and other body fluids for correlation with viable microbe cells from Bacteremia and Fungemia samples, such improved methods provided by the invention being particularly advantageous for the diagnosis of septicemia.

Further objectives and advantages of the present invention will be apparent from the following description of preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows, in table form, the results of experiments conducted to compare filter-bead mill-in situ microbe lysis and analyte analysis via DNA Polymerase (PolMA), and genomic DNA via quantitative gene specific PCR.

Figure 2 shows an illustration in diagram form of a strategy for detection of microbes in lysates according to the invention.

Figure 3 shows flow diagrams illustrating that the addition of trypsin and DNase enables significant reduction of clogging observed during the processing of two "difficult" clinical samples in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Although the present invention has been described, the following examples are also provided by way of specific illustration of embodiments of the invention and for purposes of clarity of understanding. It will be readily apparent to those of ordinary skill in the art, in light of the teachings of this invention as set forth herein, that certain changes and modifications may be made to these embodiments thus described without departing from the spirit or scope of the invention.

A chaotropic agent, also known as chaotropic reagent and chaotrope, is a substance which disrupts the three dimensional structure in macromolecules such as proteins, DNA, or RNA, and denatures them. Chaotropic agents interfere with stabilizing inter-molecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Often structural features, as detected by means such as circular dichroism can be titrated in a chaotrope concentration-dependent fashion. Chaotropic reagents include, for example:

Urea 6 - 8 mol/l

Guanidinium chloride 6 mol/l

Lithium perchlorate 4.5 mol/l

Denaturation (biochemistry)

In addition, high generic salts can have chaotropic properties, by shielding charges and preventing the stabilization of salt bridges. Hydrogen bonding is stronger in nonpolar media, so salts, which increase the dipole moment of the solvent, can also destabilize hydrogen bonding.

Often structural features, as detected by means such as circular dichroism can be titrated in a chaotrope concentration-dependent fashion. Some examples of historically useful chaotropic reagents in biochemistry and molecular biology include: Urea 6 - 8 mol/l , guanidinium chloride 6 mol/l, lithium perchlorate 4.5 mol/l, alchohols, amines (especially quaternary amines), detergents (especially nonionic), pH change, betaine, proline, carnitine, trehalose, NP-40 and the like , as well as BSA.In accordance with the present invention the design of experiment (DoE) process has been used for optimization of effective formulation ranges and combinations of ranges of various chaotropes (mixtures or reagents, or "cocktails") to: a) denature dead cell structures such that they are easily separated from live cells based on their size (filtration) and density (centrifugation); and b) create resultant chaotrope cocktail exposed live cell separated solutions that are directly compatible with downstream analysis amplification assays, such as PCR and the live cell derived endogenous proteins, and that maintain their measurable biochemical activities. Effectively the chaotropic cocktails will be optimized to differentiate live from dead cells based on the differential membrane integrity thereof, maintaining live cell endogenous protein activities for viability correlation analysis.

Sample Preparation:

Preferential blood cell lysis conditions yield preferential homogenization of blood cells from blood-microbe mixtures such as found in septicemia blood culture samples. Homogenization needs to occur at a sufficient level (creating a fluid) which enables passage of unwanted blood cells fluid through a filter from the Feed side (retaining desired microbe cells) through to the filtrate side effectively separating these two populations. These lysis conditions would enable the microbial cells to remain intact and thus enable rapid/sensitive filter-based separation of homogenized blood cells by retaining microbe cells.

In accordance with the present invention, differential blood cell Lysis and sufficient homogenization of their resulting cell debris are employed to reduced blood cells down to a fluid level enabling differential filterability where the filter retains microbes on the Feed side, thus separating the intact microbes, for subsequent sterile fluids analyses. Filter pore sizes known to those in the art as pore sizes measuring between 0.45um, 0.22um, 0.1um in diameter should be sufficient. However these effective pore sizes could be both smaller than 0.1 and larger than 0.45 depending on the microbe and differential cell debris size filterability. Conditions include but are not limited to optimized combinations of detergent, proteinases, chaotrops, denaturants, and nucleases to achieve the desired effects.

Microbe specific filter-in situ is defined herein as employing physical and biochemical cell wall lysis methods while microbes are captured on the Feed side of the filter and /or subsequent microbe specific analyte assays applied in situ. Furthermore, herein "in situ" means lysis and or subsequent analysis occurs after differential separation of undesired interfering cells (i.e. Blood cells) while desired microbe cells are still retained on the Feed side of the filter. Thus it is expected that the captured microbes are likely suspended in residual Feed Filter solution used to

load and wash the filter. The physical forces employed to lyse these now separated, intact and filter-contained microbes are those common to those skilled in this art including but not limited to enzymatic cell wall digestion. Furthermore in accordance with the invention filter-in situ sonication of all microbes by direct probe contacting the residual liquid retained by surface tension on the filter side containing the separated microbes, alternatively by sonic probe contacting the opposite side of the filter from the microbes and transferring its lytic energy via through the pores not through the solid filter material. In addition, it has been surprisingly found that efficiency of filter-bead-mill in situ for microbe lysis of bacteria and yeast occurs as well in a closed microfuge tube as it does directly on the filter Feed surface after capturing microbes spiked in blood where the blood cells were differentially lysed and filter separated. In this manner filter-in situ as defined herein is an elegant simplification of septicemia sample preparation enabling more efficient processing with less manipulations, less potential for contamination, more flexible formats both manually and for automated device designs.

As used in the following examples, filtration is employed as the term is commonly used in the art, that is, a mechanical or physical operation which is used for the separation of solids from fluids (liquids or gases) by interposing a medium through which only the fluid can pass. In a typical simple filtration, oversize particles in the liquid being filtered cannot pass through the lattice structure of the filter, whereas fluid and small particles pass through, becoming filtrate.

Example 1

Experiments were conducted to compare filter-bead mill-in situ microbe lysis and analyte analysis via DNA Polymerase (PolMA), and genomic DNA via quantitative gene specific PCR. The results are presented in the tables illustrated in Figure 1 of the drawings.

Interpretation of delta Ct values must be greater than two to be considered a significant difference when comparing relative qPCR values as is done here.

Results and Conclusions:

The relative qPCR difference values between starting input microbe spikes and corresponding filter captured samples shows in general a very high % recovery of various microbes spiked into blood and then captured on the Feed side of the filter and then bead mill lysed on the feed side of the filter termed here "filter-mill in situ". Of the 14 different microbes that were measurable by PCR only four (all Candida yeasts) (28%) showed any significant PCR recovery differences. Yet for these yeasts there was an increase in measurable DNA polymerase activity from these same samples. Overall, this indicated an excellent recovery and high efficiency filter mill in situ yielding both high DNA polymerase activity and amplifiable genomic DNA. Unexpectedly, significant negative values in bold red show that filter in situ dependent PolMA in accordance with the invention can be a significant improvement standard milling in a microfuge tube.

The strategy for detection of microbes in lysates according to the invention can be summarized in the diagram appended hereto as Figure 2.

Example 2

This example of an embodiment of the invention demonstrates the suitability of the present invention for circumventing the necessity for conventional DNA isolation techniques, and for enabling microbe lysate-direct-probe-based-PCR techniques to be performed

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a. *Staphylococcus Aureus* (SA) was spiked into standard blood cultures, (Candida consensus assay, E.Coli, E faecium) followed by WBC detergent + base lysis, pelletizing, and washing.

b. It was found that after direct lysate PCR using both TaqMan probe and SYBR that the direct probe procedure in accordance with the invention was in each case superior in terms of higher tolerance of % lysate in PCR (up to 17% with no inhibition detected from at least 5000 microbes in 5ul mill lysate, in 30ul PCR. Blood culture positive bottles will contain ~4000 microbes /ml of culture, placing 2ml in prep yields 8000 microbes/50ul lysate of which 5ul in 30ul PCR reaction = 160 microbes in PCR (Upper BC level required assay tolerance). It is presently estimated that the limit of detection of BC to be 500 microbes/ bottle or 10 microbes /ml, therefore 5ul = 2. If 10 microbes/bottle (common), then 5ul = 0.2 microbes then requiring 6 doubling generations to = 640/bottle, which can be detectable.

c. Accordingly, it has been shown in accordance with the invention that SA microbes run through (chaotrope + detergent) MolYsis buffer and DNase treatment, followed by 1 TE pellet & wash are compatible with mill-direct probe PCR. The novel improved methods of the invention were shown by the

improvements in the blood mill direct system utilized, in terms of sensitivity and tolerance of % blood over the conventional art, by comparing blood culture bead mill systems without denaturants (the Becton Dickinson Staph S/R kit, commercially available from Becton Dickinson), where only 1/10e6th of sample is in PCR, to the system provided by the improvements of the present invention with denaturants (DoE: guanidine/tween, trition/NaOH, tween/trition etc.)

Example 3

Further in experiments during the development of the invention, it was demonstrated that the addition of trypsin and DNase enables significant reduction of clogging observed during the processing of two "difficult" clinical samples in accordance with the present invention, as presented in the flow diagrams shown in Figure 3 appended hereto.

It will be appreciated by those of ordinary skill in the art that the broad fundamental principles and teachings of the present invention are capable of being applied to optimize all variations of denaturant-enabled-crude lysate (bead mills & ultrasonics)-direct-probe/SYBR-PCR analysis of various biological tissue samples (including, but not limited to, blood, body fluid, and soft tissues) for not only SA as specifically described above, but also for various pathogens, such as any bacteria, fungi, virus, parasites, etc.

The above examples also show that the practice of the methods provided by the invention can efficiently suppress signals from killed cells in defined mixtures or in an environmental sample spiked with defined mixtures of live and killed cells. It is also worthwhile to note that treatment of samples in accordance with the invention might be a good way to exclude membrane-compromised cells from analysis.

Summarizing the above, this invention provides novel methods enabling fast and easy-to-perform pre- treatment of a bacterial population before further downstream analyses. Although the potential numerous applications of the invention will be appreciated by those skilled in the art, the methods provided by the invention may have a great impact on DNA-based diagnostics in various fields, including pathogen diagnostics, bioterrorism and microbial ecology.

In the practice of a preferred embodiment of the invention, it will be apparent that because cells don't grow, any PCR measurement of at least two separate time points using separate but equal aliquots from a single blood culture that shows a significant increase in a microbe target signal must be due to microbe growth, thereby indicating the presence of viable microbes (disregarding contamination effects). It is to be appreciated that non-growth based single point positive PCR analysis of blood will indicate the presence of a viable microbe when all dead cell DNA has been eliminated, prior to viable microbe lysis and PCR setup – baring any PCR process induced contamination. This can be demonstrated by by DNasing and Washing away dead cell DNA.

Although specific references are made herein to PCR, It is further to be appreciated that the improvements of the present invention are not limited to PCR or similar methodologies. Amplification assays contemplated for use in the present invention include, but are not limited to, other well-known nucleic-acid based techniques such as DNA amplification assays, PCR assays incorporating thermostable polymerases, and isothermal amplifications methods. It is to be appreciated that one skilled in the art may conceive of various suitable amplification methods that will be useful in the practice of the present invention, and that therefore the invention is not intended to be limited thereby.

It is to be appreciated that the present invention has applications in any and all methods, procedures and processes involving DNA diagnostics. Examples of such applications include but are not limited to those involving food, water safety, bioterrorism, medical/medicines and/or anything involving pathogen detection. In the food industry, the present invention can be used to monitor the efficacy of preservatives. The method of the invention has the potential to be applied to all cells. Although bacterial cells are exemplified in the example, one of ordinary skill in the art can easily see that the methods of the invention can be applied to many other cell types. The invention can also be used for the identification of substances that can disrupt membranes and/or kill cells, e.g. bacterial cells. The identification of new disinfectants and/or antibiotics are now a priority since multidrug resistance organisms have flourished and spread in health institutions and patients.

It will further be appreciated that the methods of the invention, in combination with quantitative PCR as a tool, can quickly and successfully identify the impact of a disinfectant and/or antibiotic without having to spend time culturing the cells and waiting for growth. In some instances, organisms can take days to weeks to culture, and thus it can take significant time to see if the candidate substance has been able to kill cells, like microorganisms. In other instances, certain organisms will not grow in cell culture, therefore making it difficult to determine if a substance was effective. Thus, applying the novel methods of the invention can save time and resources for identification of novel disinfectants and/or antibiotics.

A further advantage of the novel methods according to the invention is ease of use. For example, using these methods, large amounts of samples can easily be tested for the presence of viable cells, e.g. bacteria. For example, samples may be tested for the presence of potentially

live bacteria with intact cell membranes. In another embodiment, environmental samples may be tested for the presence of viable cells, e.g. bacteria. These samples may be, for example, collected from soil or be parts of plants. The methods according to the invention can further be used for testing of treated waste water both before and after release.

The methods according to the invention may further be used for testing medicinal samples, e.g., stool samples, blood cultures, sputum, tissue samples (also cuts), wound material, urine, and samples from the respiratory tract, implants and catheter surfaces.

Another field of application of the methods according to the invention can be the control of foodstuffs. In other embodiments, the food samples are obtained from milk or milk products (yogurt, cheese, sweet cheese, butter, and buttermilk), drinking water, beverages (lemonades, beer, and juices), bakery products or meat products. The method of the invention can determine if preservatives in the food or antimicrobial treatment of food (such as pasteurization) has prevented cell growth. A further field of application of the method according to the invention is the analysis of pharmaceutical and cosmetic products, e.g. ointments, creams, tinctures, juices, solutions, drops, etc.

The methods of the invention solve the problem of long incubation times (in the range of days) making the older methods unsuitable for timely warning and preventive action. In addition, modern PCR based methods can give false positive results (testing positive for an organism although the organism is not viable). Moreover, research has recently discovered that some organisms can, under certain circumstances, lose the ability to replicate although they are still viable. These 'viable but not culturable' (VBNC) bacteria cannot be detected using traditional cultivation but might regain their ability to grow if transferred to a more appropriate

environment. These drawbacks are solved by applying molecular approaches based on the detection of genetic material/DNA of these organisms in combination with the methods of the invention. Thus, quick and accurate results regarding viable organisms in a sample, e.g. contaminated water, sewage, food, pharmaceuticals and/or cosmetics, can prevent contaminated products from being released to the public. The methods of the invention can save resources, by minimizing false positives (testing positive for a pathogen although the pathogen is not viable) and rapid testing of samples, as compared to the current time consuming methods.

In addition, the methods of the invention can identify potentially viable members of a microbial community for ecological studies, health of specific soils for agricultural and/or ecological systems. Traditionally identifying a bacterial community has been performed using cultivation-based approaches or plate counts. The more colonies that are counted, the more bacteria are estimated to be in the original sample roblems, however, arise from sometimes long incubation times (in the range of days) making this method unsuitable for timely and accurate results. These drawbacks are utilizing the methods of the invention.

Non-limiting examples of bacteria that can be subjected to analysis using the methods of the invention or to detect potential viability in a sample using the method of the invention comprise, in addition to SA as previously described: B. pertussis, Leptospira pomona, S. paratyphi A and B, C. diphtheriae, C. tetani, C. botidinum, C. perfringens, C.feseri and other gas gangrene bacteria, B. anthracis, P. pestis, P. multocida, Neisseria meningitidis, N. gonorrheae, Hemophilus influenzae, Actinomyces {e.g., Norcardia), Acinetobacter, Bacillaceae {e.g., Bacillus anthrasis), Bacteroides {e.g., Bacteroides fragilis), Blastomycosis, Bordetella, Borrelia {e.g., Borrelia burgdorferi), Brucella, Campylobacter, Chlamydia, Coccidioides,

Corynebacterium {e.g., Corynebacterium diptheriae), E. coli {e.g., Enterotoxigenic E. coli and Enterohemorrhagic E. coli), Enterobacter (e.g. Enter obacter aerogenes), Enterobacteriaceae (Klebsiella, Salmonella (e.g., Salmonella typhi, Salmonella enteritidis, Serratia, Yersinia, Shigella), Erysipelothrix, Haemophilus (e.g., Haemophilus influenza type B), Helicobacter, Legionella (e.g., Legionella pneumophila), Leptospira, Listeria (e.g., Listeria monocytogenes), Mycoplasma, Mycobacterium (e.g., Mycobacterium leprae and Mycobacterium tuberculosis), Vibrio (e.g. , Vibrio cholerae), Pasteurellacea, Proteus, Pseudomonas (e.g., Pseudomonas aeruginosa), Rickettsiaceae, Spirochetes (e.g., Treponema spp., Leptospira spp., Borrelia spp.), Shigella spp., Meningiococcus, Pneumococcus and all Streptococcus (e.g., Streptococcus pneumoniae and Groups A_3 B, and C Streptococci), Ureaplasmas. Treponema pollidum, Staphylococcus aureus, Pasteurella haemolytica, Corynebacterium diptheriae toxoid, Meningococcal polysaccharide, Bordetella pertusis, Streptococcus pneumoniae, Clostridium tetani toxoid, and Mycobacterium bovis. The above list is intended to be merely illustrative and by no means is meant to limit the invention to detection to those particular bacterial organisms.

A particularly preferred embodiment of the present invention utilizes PCR. General procedures for PCR are taught in U.S. Pat. No. 4,683,195 (Mullis, et al.) and U.S. Pat. No. 4,683,202 (Mullis, et al.). However, optimal PCR conditions used for each amplification reaction are generally empirically determined or estimated with computer software commonly employed by artisans in the field. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, Mg^{2+}, pH, and the relative concentration of primers, templates, and deoxyribonucleotides. Generally, the template nucleic acid is denatured by heating to at least about 95°C for 1 to 10 minutes prior to the polymerase reaction. Approximately 20-99 cycles of amplification are executed using denaturation at a range of 90^0C

to 96°C for 0.05 to 1 minute, annealing at a temperature ranging from 48°C to 72°C for 0.05 to 2 minutes, and extension at 68°C to 75°C for at least 0.1 minute with an optimal final cycle. In one embodiment, a PCR reaction may contain about 100 ng template nucleic acid, 20 uM of upstream and downstream primers, and 0.05 to 0.5 mm dNTP of each kind, and 0.5 to 5 units of commercially available thermal stable DNA polymerases.

A variation of the conventional PCR is reverse transcription PCR reaction (RT-PCR), in which a reverse transcriptase first coverts RNA molecules to single stranded cDNA molecules, which are then employed as the template for subsequent amplification in the polymerase chain reaction. Isolation of RNA is well known in the art. In carrying out RT-PCR, the reverse transcriptase is generally added to the reaction sample after the target nucleic acid is heat denatured. The reaction is then maintained at a suitable temperature (e.g. 30-45°C) for a sufficient amount of time (10-60 minutes) to generate the cDNA template before the scheduled cycles of amplification take place. One of ordinary skill in the art will appreciate that if a quantitative result is desired, caution must be taken to use a method that maintains or controls for the relative copies of the amplified nucleic acid. Methods of "quantitative" amplification are well known to those of skill in the art. For example, quantitative PCR can involve simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction.

Another alternative of PCR is quantitative PCR (qPCR). qPCR can be run by competitive techniques employing an internal homologous control that differs in size from the target by a small insertion or deletion. However, non-competitive and kinetic quantitative PCR may also be

used. Combination of real-time, kinetic PCR detection together with an internal homologous control that can be simultaneously detected alongside the target sequences can be advantageous.

Primers for PCR, RT-PCR and/or qPCR are selected within regions or specific bacteria which will only amplify a DNA region which is selected for that specific organism. Alternatively, primers are selected which will hybridize and amplify a section of DNA which is common for all organisms. Primer selection and construction is generally known in the art. In general, one primer is located at each end of the sequence to be amplified. Such primers will normally be between 10 to 35 nucleotides in length and have a preferred length from between 18 to 22 nucleotides. The smallest sequence that can be amplified is approximately 50 nucleotides in length (e.g., a forward and reverse primer, both of 20 nucleotides in length, whose location in the sequences is separated by at least 10 nucleotides). Much longer sequences can be amplified. One primer is called the "forward primer" and is located at the left end of the region to be amplified. The forward primer is identical in sequence to a region in the top strand of the DNA (when a double- stranded DNA is pictured using the convention where the top strand is shown with polarity in the 5' to 3' direction). The sequence of the forward primer is such that it hybridizes to the strand of the DNA which is complementary to the top strand of DNA. The other primer is called the "reverse primer" and is located at the right end of the region to be amplified. The sequence of the reverse primer is such that it is complementary in sequence to, i.e., it is the reverse complement of a sequence in, a region in the top strand of the DNA. The reverse primer hybridizes to the top end of the DNA. PCR primers should also be chosen subject to a number of other conditions. PCR primers should be long enough (preferably 10 to 30 nucleotides in length) to minimize hybridization to greater than one region in the template. Primers with long runs of a single base should be avoided, if possible. Primers should preferably have a percent G+C content

of between 40 and 60%. If possible, the percent G+C content of the 3' end of the primer should be higher than the percent G+C content of the 5' end of the primer. Primers should not contain sequences that can hybridize to another sequence within the primer (i.e., palindromes). Two primers used in the same PCR reaction should not be able to hybridize to one another. Although PCR primers are preferably chosen subject to the recommendations above, it is not necessary that the primers conform to these conditions. Other primers may work, but have a lower chance of yielding good results.

PCR primers that can be used to amplify DNA within a given sequence can be chosen using one of a number of computer programs that are available. Such programs choose primers that are optimum for amplification of a given sequence (i.e., such programs choose primers subject to the conditions stated above, plus other conditions that may maximize the functionality of PCR primers). One computer program is the Genetics Computer Group (GCG recently became Accelrys) analysis package which has a routine for selection of PCR primers.

The oligonucleotide primers and probes disclosed below can be made in a number of ways. One way to make these oligonucleotides is to synthesize them using a commercially-available nucleic acid synthesizer. A variety of such synthesizers exists and is well known to those skilled in the art.

Another alternative of PCR useful in connection with the invention is isothermal nucleic acid amplification assay for the detection of specific DNA or RNA targets. Non-limiting examples for isothermal amplification of nucleic acids are homogeneous real-time strand displacement amplification, Phi29 DNA polymerase based rolling circle amplification of

templates for DNA sequencing, rolling-circle amplification of duplex DNA sequences assisted by PNA openers or loop-mediated isothermal amplification of DNA analytes.

Nucleic acid may also be detected by hybridization methods. In these methods, labeled nucleic acid may be added to a substrate containing labeled or unlabeled nucleic acid probes. Alternatively, unlabeled or unlabeled nucleic acid may be added to a substrate containing labeled nucleic acid probes. Hybridization methods are disclosed in, for example, Micro Array Analysis, Marc Schena, John Wiley and Sons, Hoboken N.J. 2003.

Methods of detecting nucleic acids can include the use of a label. For example, radiolabels may be detected using photographic film or a phosphoimager (for detecting and quantifying radioactive phosphate incorporation). Fluorescent markers may be detected and quantified using a photodetector to detect emitted light (see U.S. Pat. No. 5,143,854, for an exemplary apparatus). Enzymatic labels are typically detected by providing the enzyme with a substrate and measuring the reaction product produced by the action of the enzyme on the substrate. Colorimetric labels are detected by simply visualizing the colored label. In one embodiment, the amplified nucleic acid molecules are visualized by directly staining the amplified products with a nucleic acid-intercalating dye. As is apparent to one skilled in the art, exemplary dyes include but not limited to SYBR green, SYBR blue, DAPI, propidium iodine, Hoeste, SYBR gold and ethidium bromide. The amount of luminescent dyes intercalated into the amplified DNA molecules is directly proportional to the amount of the amplified products, which can be conveniently quantified using a FluroImager (Molecular Dynamics) or other equivalent devices according to manufacturers' instructions. A variation of such an approach is gel electrophoresis of amplified products followed by staining and visualization of the selected

intercalating dye. Alternatively, labeled oligonucleotide hybridization probes (e.g. fluorescent probes such as fluorescent resonance energy transfer (FRET) probes and colorimetric probes) may be used to detect amplification. Where desired, a specific amplification of the genome sequences representative of the biological entity being tested, may be verified by sequencing or demonstrating that the amplified products have the predicted size, exhibit the predicted restriction digestion pattern, or hybridize to the correct cloned nucleotide sequences.

The present invention also comprises kits. For example, the kit can comprise primers useful for amplifying nucleic acid molecule corresponding to organisms specifically or generally, buffers and reagents for isolating DNA, and reagents for PCR. The kit can also include detectably labeled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to organisms of interest. The kit can also contain a control sample or a series of control samples which can be assayed and compared to a test sample contained. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit

The contents of all references, patents and published patent applications cited throughout this application, are incorporated herein by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom as modifications will be obvious to those skilled in the art. It is not an admission that any of the information provided herein is

prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

Also, while certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments, and any such limitations are contained only in the following claims.

2011352333 10 Nov 2016

The claims defining the invention are as follows:

1. A method for detecting viable microbe cells in a mixture containing viable and dead microbe cells, wherein the method comprises:

   (a) eliminating amplification assay inhibitors from the mixture by addition of one or more chaotrope;

   (b) removing dead microbe cell DNA prior to performing a nucleic acid amplification assay;

   (c) performing the nucleic acid amplification assay, wherein obtaining a positive non-contaminated result from the assay indicates that viable cells are present;

   wherein said one or more chaotrope denatures dead cell structures and eliminates amplification assay inhibitors.

2. The method of claim 1, wherein the amplification assay is a PCR assay.

3. The method of claim 2, wherein the PCR assay is a quantitative PCR (qPCR) assay.

4. The method of any one of claims 1 to 3, wherein the mixture comprises blood or other body fluids.

5. The method of claim 2 or 3, wherein performing the PCR assay provides correlation with viable microbe cells from Bacteremia and Fungemia samples for the diagnosis of septicemia.

Fig. 1

Recovery Comparison of Microbe Analytes: Standard Bead Mill Vs. Novel Filter-Mill-In Situ: measuring DNA Polymerase (PolMA) and qPCR.

11/30/2010

Blood donor	Sepsis Microbe	Actual cfu per 0.5 ml blood prep	Non-blood prep, standard bead mill-PolMA Ct	0.2uM filter based-blood prep-PolMA Ct	Non-blood prep, standard bead mill-gene specific Ct	0.2uM filter based-blood prep-gene specific Ct	DNA Polymerase Activity, delta Ct	Genomic DNA via qPCR, delta Ct
W.W.	S. aureus	1200	34.6	35.1	31.3	31.58	1.5	0.2
W.W.	E. coli	1750	35.7	36.1	33.08	33.6	0.4	0.5
W.W.	K. pneumoniae	900	35	36.2	34.03	35.13	1.2	0.5
W.W.	S. pneumoniae	365	33.8	36.2	31.8	34.11	2.4	2.3
W.W.	E. faecalis	1400	37.2	37.1	33.97	33.77	-0.1	0.3
W.W.	E. faecium	1400	35	36.7	33.6	31.46	0.7	-0.1
W.W.	S. pyogenes	850	35	37.2	33.42	34.22	2.2	0.8
W.W.	S. epidermidis	1500	35.4	39.2	31.9	32.55	3.8	0.6
W.W.	S. agalactiae	1400	37.8	38.5	30.05	31.31	1.7	1.3
W.W.	P. aeruginosa	570	33.9	37.2	NA	NA	3.3	
W.W.	No spike	NA	NA	33.2	NA	NA		

12/2/2010

Blood donor	Sepsis Microbe	Actual cfu per 0.5 ml blood prep	Non-blood prep, standard bead mill-PolMA Ct	0.2uM filter based-blood prep-PolMA Ct	Non-blood prep, standard bead mill-gene specific Ct	0.2uM filter based-blood prep-gene specific Ct	DNA Polymerase Activity, delta Ct	Genomic DNA via qPCR, delta Ct
D.R.	A. baumannii	800	37.3	37.7	No assay available	No assay available	0.4	
D.R.	E. cloacae	2200	36.5	35.2	No assay available	No assay available	0.7	
D.R.	H. influenziae	NA	31.7	33.4	No assay available	No assay available	1.7	
D.R.	C. albicans	90000	32	24.9	24.99	25.6	-7.1	1.6
D.R.	C. tropicalis	48000	28.5	27.2	22.92	25.2	-2.3	2.3
D.R.	C. krusei	38000	27.9	28.3	28.02	31.04	-1.6	3.8
D.R.	C. parapsilosis	110000	30.1	28.9	23.86	26.74	-1.2	2.9
D.R.	C. glabrata	180000	26.2	25.5	20.94	25.74	-2.7	5.7
D.R.	No spike	NA	36.5	33.2	NA	NA	-3.3	

Fig. 2

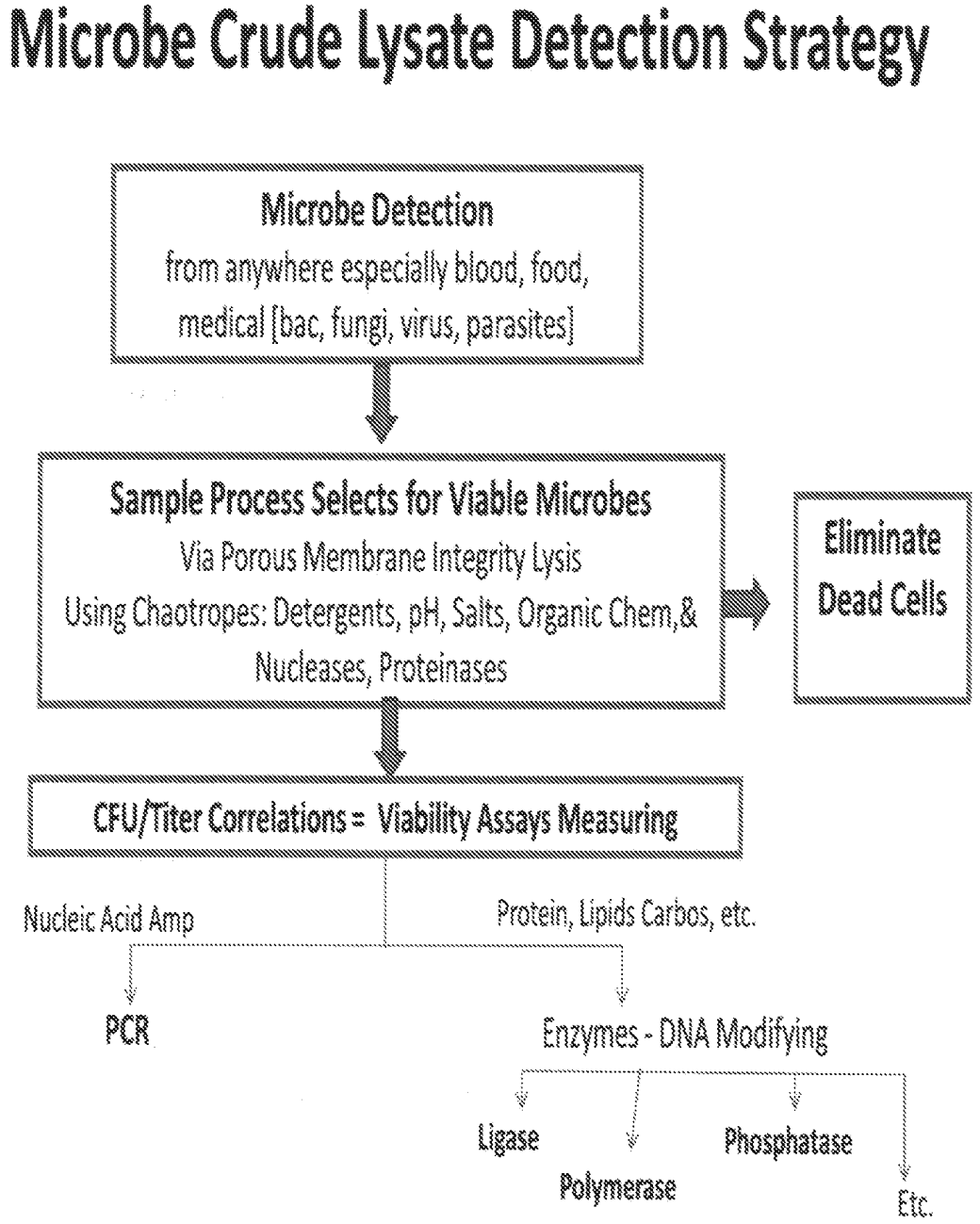
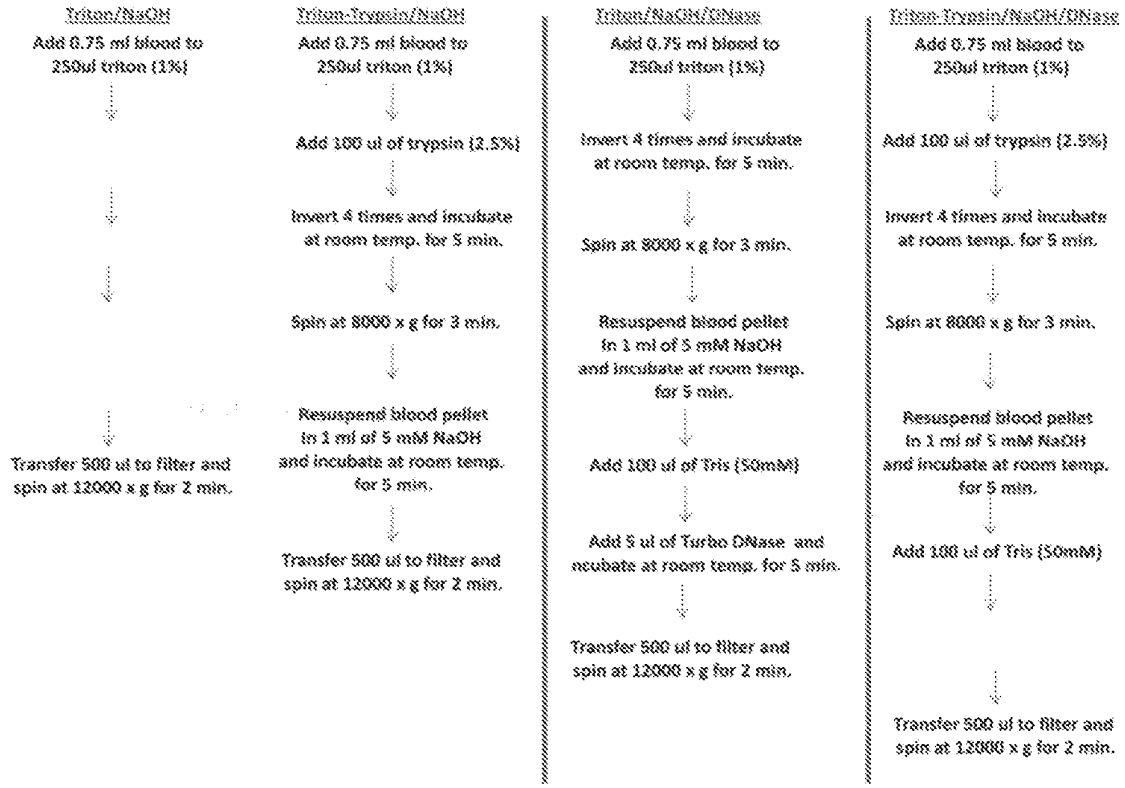

Microbe Crude Lysate Detection Strategy
Microbe Detection
from anywhere especially blood, food,
medical [bac, fungi, virus, parasites]
Sample Process Selects for Viable Microbes
Via Porous Membrane Integrity Lysis
Using Chaotropes: Detergents, pH, Salts, Organic Chem,&
Nucleases, Proteinases
Eliminate
Dead Cells
CFU/Titer Correlations = Viability Assays Measuring
Nucleic Acid Amp
Protein, Lipids Carbos, etc.
PCR
Enzymes - DNA Modifying
Ligase
Polymerase
Phosphatase
Etc.

Fig. 3


Triton/NaOH
Add 0.75 ml blood to 250ul triton (1%)
Transfer 500 ul to filter and spin at 12000 x g for 2 min.
Triton-Trypsin/NaOH
Add 0.75 ml blood to 250ul triton (1%)
Add 100 ul of trypsin (2.5%)
Invert 4 times and incubate at room temp. for 5 min.
Spin at 8000 x g for 3 min.
Resuspend blood pellet in 1 ml of 5 mM NaOH and incubate at room temp. for 5 min.
Transfer 500 ul to filter and spin at 12000 x g for 2 min.
Triton/NaOH/DNase
Add 0.75 ml blood to 250ul triton (1%)
Invert 4 times and incubate at room temp. for 5 min.
Spin at 8000 x g for 3 min.
Resuspend blood pellet in 1 ml of 5 mM NaOH and incubate at room temp. for 5 min.
Add 100 ul of Tris (50mM)
Add 5 ul of Turbo DNase and incubate at room temp. for 5 min.
Transfer 500 ul to filter and spin at 12000 x g for 2 min.
Triton-Trypsin/NaOH/DNase
Add 0.75 ml blood to 250ul triton (1%)
Add 100 ul of trypsin (2.5%)
Invert 4 times and incubate at room temp. for 5 min.
Spin at 8000 x g for 3 min.
Resuspend blood pellet in 1 ml of 5 mM NaOH and incubate at room temp. for 5 min.
Add 100 ul of Tris (50mM)
Transfer 500 ul to filter and spin at 12000 x g for 2 min.