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(54) **Title**: VIABILITY ANALYSIS OF PROTOZOA USING POLYMERASE CHAIN REACTION (PCR)

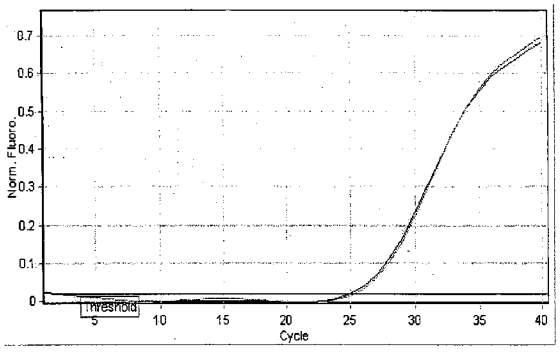

Figure 6

(57) **Abstract**: The present invention relates to a method for detecting viable protozoa cells in a sample fluid comprising the steps of: (i) contacting a sample fluid with a phenanthridium compound capable of preferentially penetrating dead or membrane-compromised cells over viable and/or substantially intact cells to intercalate with at least one nucleic acid molecule to; (ii) exposing the sample fluid to a light source to substantially covalently bind nucleic acid molecules of dead or membrane-compromised cells to the phenanthridium compound to form a reacted sample; (iii) separating the cells from any excess phenanthridium compound; (iv) lysing the viable and/or substantially intact cells to release nucleic acid molecules into solution; and (v) performing a PCR on the nucleic acid solution with PCR primers specific to the protozoa, wherein detection of amplification indicates the presence of viable and/or substantially intact protozoa cells.

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Viability analysis of protozoa using polymerase chain reaction (PCR)

Field of the invention

The present invention relates to the detection of organisms, for example, in environmental sampling, food and water safety, pathogen detection and disease control. The invention in particular relates to the detection and/or differentiation of viable cells, for example protozoa.

Background of the invention

The detection of viable or live organisms presents an important challenge for many applications. For example, in the case of pathogenic microorganisms, it is important to identify viable or live cells as these cells are metabolically active and/or reproductive with the potential to infect and cause diseases. In particular, the ability to determine the concentration of viable cells is important.

For example, detection of viable *Cryptosporidium* is very crucial for water monitoring. *Cryptosporidium* was once an emerging pathogen but now it is an established human infectious waterborne and food borne pathogen. A major outbreak in 1993 in Wisconsin, Milwaukee, USA affected an estimated 403,000 people (Kaminski *et* al., 1984). In the event of such outbreaks it may be vital to determine the concentration of viable *Cryptosporidium* oocysts present in the drinking water sample in order to make accurate risk estimations. Current methods of *Cryptosporidium* contamination of water networks are done by USEPA method 1622 and method 1623. These methods are based on microscopy staining of concentrated samples but do not provide any information on the viability of the oocysts.

However, traditional microscopy methods alone are not able to detect viable and/or live organisms, such as protozoa, for example *Cryptosporidium parvum*. In recent years, the methods adopted for testing viability of *Cryptosporidium*

parvum oocysts in water samples are largely based on staining with fluorogenic dyes like PI/DAPI with microscopic observation, in-vitro excystation, cell culturing and animal infectivity methods.

With the fluorogenic dyes (DAPI & PI) method, propidium iodide (PI) and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) are fluorescent dyes used in the most commonly adopted method for assessment of survival of *C. parvum* oocysts under various environmental pressures. The blue-fluorescent DAPI nucleic acid stain preferentially stains DNA (Gasser *et al.*, 1999). PI is also a fluorescent dye which can penetrate into the dead cells walls with compromised membrane permeability. Sporozoite nuclei, which take up DAPI but fail to stain with PI, are viable (i.e. $DAPI^+$, PI^-) and termed ghost cells or empty cells, while cells that stain with both fluorochromes are nonviable (i.e. $DAPI^-$, PI^-). This method requires skilled labour, a fluorescence microscope and is labour intensive and a lengthy procedure (i.e. time consuming). Moreover, this dye permeability assay was found to be unreliable and was not able to indicate the viability of the oocysts during oocysts disinfection studies (Korich *et al.*, 1990).

In the *in* vitro excystation method to determine oocysts viability, the host body environment is simulated, and this includes creating a variety of conditions like addition of bile salts, pancreatic juices and setting up appropriate environmental conditions. After excystation, the oocysts are microscopically observed for released or partly released sporozoites. However, this method is effective only for high concentrations of oocysts, and is not feasible for application to testing of environmental water samples where the oocysts concentration is very low.

With mammalian cell culturing, cultured cell lines are infected by oocysts samples and observed for parasite antigen after 24-48 hours by Foci Detection method. The success of mammalian cell culturing depends upon various factors like the cell lines used, the purity of oocysts suspension, pre-incubation excystation treatment and centrifugation techniques of the sample (Carey *et al.*,

2004). Moreover, this technique also requires specialized equipment and experienced personnel to handle and maintain cultures, which are not applicable for routine testing of water samples.

Animal infectivity methods are considered the most direct method to investigate the viability of oocysts. Samples are directly injected into live mice, followed by observation of diarrheal symptoms and shedding of oocysts in faeces. Peeters *et al.*, 1989 found that 1000 oocysts per mouse were required to induce 100% infection and therefore the method is not easily adaptable to routine environmental testing. Moreover, animal testing is very expensive and time consuming.

In Cell Culture-PCR, seeded oocysts are concentrated by filtration and centrifugation, and inoculated onto a human colon carcinoma (Caco-2) cell line (Rochelle *et al.*, 1997). Intensive labour is required for the growth and maintenance of the cell monolayer and the prolonged incubation period represent significant disadvantages of the cell culture technique, particularly in confirming a potential waterborne outbreak where time is of essence. However, the technique remains a good substitute for animal infectivity models.

It is desirable to develop efficient methods of differentiating viable cells.

Summary of the invention

The present invention relates to a method for detecting viable cells. This method importantly concentrates, differentiates, separates and detects the viable cells. According to a first exemplary aspect, the method for detecting viable protozoa in a sample fluid comprises the steps of:

i) contacting a sample fluid with a phenanthridium compound capable of preferentially penetrating dead or membrane-compromised cells over viable and/or substantially intact cells to intercalate with at least one nucleic acid molecule;

(ii) exposing the sample fluid to a light source to substantially covalently bind nucleic acid molecules of dead or membrane-compromised cells to the phenanthridium compound to form a reacted sample;

(iii) separating the cells from any excess phenanthridium compound;

(iv) lysing the viable and/or substantially intact cells to release nucleic acid molecules into solution; and

(v) performing a PCR on the nucleic acid solution with PCR primers specific to the protozoa, wherein detection of amplification indicates the presence of viable and/or substantially intact protozoa cells.

Any suitable method for separating the cells from excess phenanthridium compound may be used. In particular, the cells may be separated by using a filter and then recovered from the filter for further analysis.

Accordingly, in a second exemplary aspect, there is provided a method for detecting viable protozoa in a sample fluid comprising the steps of:

(i) contacting a sample fluid with a phenanthridium compound capable of preferentially penetrating dead or membrane-compromised cells over viable and/or substantially intact cells to intercalate with at least one nucleic acid molecule;

(ii) exposing the sample fluid to a light source to substantially covalently bind nucleic acid molecules of dead or membrane-compromised cells to the phenanthridium compound;

(iii) separating the cells of the sample from any excess phenanthridium compound using a filter;

(iv) recovering the cells from the filter;

(v) lysing the viable and/or substantially intact cells to release the nucleic acid molecules into solution; and

(vi) performing a PCR on the nucleic acid solution with PCR primers specific to the protozoa, wherein detection of amplification indicates the presence of viable and/or substantially intact protozoa cells.

Brief description of the figures

The invention will be better understood with reference to the drawings, in which:

Figure 1 depicts an example of a filter apparatus.

Figure 2 depicts a device for exposing a tube to a blue LED light.

Figure 3(a) depicts a real-time PCR reaction for quantifying DNA to determine the extraction efficiency and 3(b) depicts the real-time standard curve for *C. parvum* for the logarihmic amplification plots for 10^2 , 5 x 10^3, 10^4 and 10^6 oocysts per tube (x-axis: no. of cells, y-axis: Crossing threshold, Ct value).

Figure 4 depicts a real-time PCR result showing the effect of PMA on amplification of DNA from inactive *C. parvum* cells. Plot 1 is for inactive *C. parvum* without PMA treatment with a Ct value of 26.99; Plot 2 is for inactive *C. parvum* treated with 12.5 μM PMA with a Ct value of 32.11; Plot 3 is for inactive *C. parvum* treated with 25 μM PMA; Plot 4 is for *C. parvum* treated with 50 μM PMA.

Figure 5 depicts a real-time PCR result showing the effect of PMA on amplification of DNA from viable *C. parvum* cells. Plot 1 is for viable *C. parvum* without PMA treatment, with a Ct value of 24.95; Plot 2 is for inactive *C. parvum* treated with 12.5 μM PMA with a Ct value of 25.04; Plot 3 is for inactive *C. parvum* treated with 25 μM PMA with a Ct value of 26.17; Plot 4 is for *C. parvum* treated with 50 μM PMA with a Ct value of 26.43.

Figure 6 depicts a real-time PCR result showing the amplification from 5000 viable *C. parvum* oocysts without PMA treatment and a mixture of 5000 viable and 5000 inactive *C. parvum* oocysts with 25 μM PMA. The amplification from 5000 viable *C. parvum* gave a Ct value of 25.02 and the amplifcation from the mixture gave a Ct value of 25.30,

Figure 7 shows the agarose gel electrophoresis of the real-time PCR of Figure 6. Lane 1: 100 bp DNA ladder; Lane 2: DNA extraction from 5000 viable *C. parvum* oocysts without PMA treatment; Lane 3: DNA extraction from a mixture of 5000 viable and 5000 inactive *C. parvum* oocysts with 25 μM PMA.

Detailed description of the invention

The present invention relates to a method to detect viable protozoa in a sample fluid. The sample fluid is contacted with a phenanthridium compound capable of preferentially penetrating dead or membrane-compromised cells over viable and/or substantially intact cells to intercalate with at least one nucleic acid molecule.

The phenanthridium compound may be any phenanthridium compound capable of preferentially penetrating dead or memberane-compromised cells over viable and/or substantially intact cells. After penetrating the dead or membrane-compromised cells, the phenanthridium compound intercalates with at least one nucleic acid molecule and covalently binds with the nucleic acid molecule on exposure to a light source. In particular, the compound propidium monoazide (PMA) may be used. The light source may be a light emitting diode (LED). In particular, the light source may emit blue light to enable the reaction to occur. Covalently bound nucleic acid molecules from dead and/or membrane-compromised cells will not amplify during PCR.

After exposure to the light source, the viable cells are separated from any excess phenanthridium compound using a filter. The viable cells are then recovered from the filter.

Any filter may be used to separate the viable cells from excess phenanthridium compound. The filter may comprise a filter membrane with pores of smaller size than the target protozoa to be detected. For example, for Cryptosporidium which are in the range of 3-6 μm, a suitable pore size may be ~2 μm.

Any conventional filter may be used, for example, including but not limited to cellulose ester filters, polycarbonate filters and the like. In particular, filter membranes with a uniform pore size may be used. An example of a filter membrane with uniform pore size which may be used is described in WO 2010/110739, the entire contents of which is herein incorporated by reference.

The filter membrane may be contained in a filter apparatus for the filtration. An example of a conventional filter apparatus that may be used for filtration is illustrated in Figure 1. The filter apparatus comprises a lid 1 with an inlet 2, a membrane filter 3 on a filter support 4 and an outlet 5. Another example of a filter apparatus suitable for the invention is described in WO 2010/110739.

After filtration, the trapped protozoa are recovered from the filter membrane and collected for further analysis. Recovery of cells from the filter membrane may be by any method. For example, the membrane filter may be immersed in a solution with agitation (e.g. tangential or lateral shaking) to dislodge the cells into the solution, and subsequently using the solution comprising the dislodged cells for further manipulation and/or analysis. As an alternative example, cells may be recovered from the membrane filter by flushing the cells from the filter membrane, for example as described in WO 2010/110739.

Separation of the viable cells from excess phenanthridium compound is performed before cell lysis. This separation step reduces the possibility of nucleic acid molecules released from the lysed cells binding to excess phenanthridium compound and not amplifying in the PCR. This separation step may also separate the viable cells from the covalently bound nucleic acid molecules of dead or membrane-compromised cells, which may inhibit or interfere with the PCR. Accordingly, only nucleic acid molecules from viable cells are amplified in the subsequent PCR step.

Lysis of the cells to release nucleic acid molecules into solution may be performed by any method, including but not limited to chemical lysis (for example using guanidine thiocyanate, proteinase K and/or any other chemical suitable for lysis), mechanical lysis, ultrasound lysis, thermolyis, freeze-thawing and electroporation. The cells are lysed to extract the nucleic acid molecules from within the cells. Following lysis, the nucleic acid may be used directly for PCR analysis. Alternatively, the nucleic acid molecules may be extracted and/or further purified before performing PCR analysis.

PCR analysis is performed with PCR primers specific to the target protozoa. Only nucleic acid molecules from the viable cells which were lysed are amplified during the PCR since the covalently bound nucleic acid molecules from dead and/or membrane-compromised cells will not be amplified. Accordingly, detection of amplification in the PCR indicates the presence of viable protozoa.

PCR analysis may be conventional PCR analysis with detection of amplification may be through agarose gel electrophoresis. Alternatively, the PCR analysis may be real-time PCR as this provides quantitative analysis.

The method of the invention may be carried out in a fluidic device and thus may be automated.

The method of the invention is suitable for detecting viable protozoa from any fluid sample, including but not limited to environmental, clinical and water samples. The fluid sample may be in liquid form. If the sample is not in liquid form, the sample may be resuspended in a suitable liquid. In particular, the method of the invention is for detecting viable protozoa, for example pathogenic protozoa in water samples, for example, reservoir and drinking water samples. Non-limiting species of viable pathogenic protozoa that may be detected include *Crytosporidium* (e.g. *Crytosporidium parvum*), *Giardia* (e.g. *Giardia duodenalis*), *Entamoeba* (e.g. *Entamoeba histolytica*).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Materials and methods

Viable and inactive *C. parvum* oocysts (bovine, Iowa isolate) were purchased from Waterborne Inc. (New Orleans, LA, PSA). The purified oocysts suspension was supplied in de-ionized water and stored at 4 °C until use. According to the manufacturer, the oocysts were inactivated by heat-killing at 72 °C water bath for 15 minutes.

Optimization of PMA treatment

PMA (Biotium Inc., USA) was added at final concentrations of 12.5 μM, 25 μM or 50 μM to sample tubes containing 1000 viable or heat-inactivated *C. parvum* oocysts in 50 μl. Samples were incubated in the dark for 5 min to allow penetration of PMA into dead or membrane-compromised cells. Samples were then exposed to a 20 W blue LED light for 5 min using the device depicted in Figure 2. The device includes a heat cooling fan 1 and heat sink 2 to cool the

device when in operation. The tube 4 is placed beneath the stand 3 as shown in Figure 2. When the device is switched on, the tube 4 is exposed to the blue light from a LED beneath the heat sink 2.

Control samples used in the study were 50 µl of viable or inactivated *C. parvum* oocysts not exposed to PMA but incubated in the dark for 5 minutes and exposed to the blue LED light for minutes. All samples and control were further concentrated by evaporation to 5 µl.

DNA isolation and quantification

C. parvum oocysts DNA was extracted using a freeze-thaw method: 5 consecutive cycles of freezing in liquid nitrogen for 1 min and thawing at 65°C for 1 min. 1% Triton X-100 were used as lysis agent. Samples were treated with proteinase K (200 µg/ml) at 56°C for 30 min. DNA was precipitated using isopropanol method. The DNA pellets were resuspended in 50 µl TE. 2.5 µl were used in PCR, the rest of the DNA was quantified using the Quant-iT picoGreen dsDNA reagent (Invitrogen Inc., USA) according to the manufacturer's instructions.

Quantitative PCR

The DNA sequence (GeneBank accession number XM_626122, SEQ ID NO: 3) uniquely present in *Cryptosporidium* species was used as the target for relative quantification of DNA extracted from *C. parvum* oocysts. Real-time quantitative PCR was performed in a total volume of 10 µl containing 1 µl extracted genomic DNA and final concentrations of 1× PCR buffer, 3 mM $MgCl_2$, 1× SYBR Green, 0.2µM of forward primer (5'- CAAACAAGGAGGAATCAG-3'; SEQ ID NO: 1), 0.2µM of reverse primer (5'- CTTCATAATCCGGCTAAA-3; SEQ ID NO: 2) using a Rotor-Gene real-time PCR machine (Qiagen, Germany). The cycling parameters were: 2 min at 95 °C followed by 40 cycles of 5 sec at 95 °C, 5 sec

at 55 °C and 5 sec at 72 °C. All the PCR products were verified by gel electrophoresis.

Result

DNA extraction and standard curve

DNA extraction efficiency from viable *C. parvum* oocysts was evaluated using Quant-iT picogreen dsDNA reagent (Invitrogen Inc., USA) and real-time PCR. Quant-iT picogreen dsDNA reagent was used according to the manufacturer's instructions to quantitate the amount of DNA extracted from viable oocysts. The amount of DNA was converted to number of oocysts based on a theoretical content of 195 fg of DNA per *C. parvum* oocyst. Each sample was then analysed by real-time PCR.

For example, the cycle threshold value was determined from each real-time PCR curve in Figure 3(a). This was plotted against the number of oocysts calculated based on the theoretical DNA content formula to give a standard curve illustrated in Figure 3(b). The measured number of oocysts of the samples was compared to the known number of oocysts used initially. The combined picogreen and real-time PCR analysis measured the DNA extraction efficiency at 93.6 %.

Inactivated *C. parvum* oocysts

PMA was added to inactivated *C. parvum* oocysts at concentrations of 12.5 µM, 25 µM and 50 µM followed by incubation and LED light exposure as describe above. DNA was isolated by a combination of lysis and freeze-thaw cycles treatment and quantified by real-time PCR. It can be seen from Figure 4 that the real-time PCR generated from the inactive *C. parvum* oocysts was totally removed by PMA treatment at concentrations of 25 µM and 50 µM.

Viable oocysts

Viable oocysts suspensions were treated with PMA at concentrations of 12.5 µM, 25 µM and 50 µM followed by incubation and LED light exposure as describe above. Real-time PCR amplification was detected at all concentrations of PMA tested including the control (Figure 5). It was observed that PMA at 25 and 50 µM may have acted as a potential PCR inhibitor as slight inhibitions of the PCRs were observed (Figure 5).

Mixture of viable and inactive *C. parvum* oocysts

PMA was added to a mixture of 5000 viable *C. parvum* oocysts and 5000 inactive *C. parvum* oocysts at a final concentration of 25 µM. Following incubation, LED exposure and DNA extraction described above, real-time PCR analysis was performed. A control sample with 5000 viable oocysts not exposed to PMA as described above was also analysed by real-time PCR. It can be seen from Figure 6 that the viable oocysts could be differentiated in the mixed samples. The similarity in the real-time PCR curves of the control and the mixed sample suggests that DNA from inactivated *C. parvum* oocysts in the mixed sample did not amplify nor affect the PCR reaction. The PCR product was verified by gel electrophoresis as shown in Figure 6.

Conclusion

PMA is a chemically modified version of propidium iodide (PI) with an azide group added to the phenanthridine ring allowing chemical cross-linkage to organic molecules upon short exposure to blue light. Photo-induced cross-linkage disables the DNA from being amplified. This study shows that PMA does not penetrate membranes of live *C. parvum* oocysts, whereas it is efficiently taken up by dead *C. parvum* oocysts with permeabilized cell membrane. In combination with rapid real-time PCR, the detection method can be completed within 20 mins. This study suggests the potential of this PMA-

real-time PCR method to be developed as a rapid and sensitive method in monitoring viable *C. parvum* contamination levels in the drinking water supply system.

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WO 2010/110739

Claims

1. A method for detecting viable protozoa in a sample fluid comprising the steps of:

(i) contacting the sample fluid with a phenanthridium compound capable of preferentially penetrating dead or membrane-compromised cells over viable and/or substantially intact cells to intercalate with at least one nucleic acid molecule;

(ii) exposing the sample fluid to a light source to substantially covalently bind nucleic acid molecules of dead or membrane-compromised cells to the phenanthridium compound;

(iii) separating the cells of the sample from any excess phenanthridium compound using a filter;

(iv) recovering the cells from the filter;

(v) lysing the viable and/or substantially intact cells to release the nucleic acid molecules into solution; and

(vi) performing a PCR on the nucleic acid solution with PCR primers specific to the protozoa, wherein detection of PCR amplification indicates the presence of viable and/or substantially intact protozoa cells.

2. The method according to claim 2, wherein the filter comprises a filter apparatus comprising a filter membrane.

3. The method according to any one of the preceding claims, wherein the filter membrane has uniformly sized pores.

4. The method according to claim 2 or 3, wherein recovering the cells from the filter comprises agitating the filter membrane to dislodge the cells into a solution or flushing the cells from the filter membrane.

5. The method according to any one of the preceding claims, wherein lysing the cells is by chemical lysis, mechanical lysis, ultrasound lysis, thermolyis, freeze-thawing or electroporation.

6. The method according to any one of the preceding claims, wherein the nucleic acid molecules are purified before PCR.

7. The method according to any one of the preceding claims, wherein the phenanthridium compound comprises propidium monoazide.

8. The method according to any one of the preceding claims, wherein the light source comprises blue light.

9. The method according to any one of the preceding claims, wherein the PCR comprises real-time PCR.

10. The method according to any one of the preceding claims, wherein the protozoa comprises *Cryptosporidium, Giardia* and *Entamoeba* species.

11. The method according to any one of the preceding claims, wherein the protozoa comprises *Cryptosporidium parvum*, *Giardia Entamoeba histolytica*.

12. The method according to claim 11, wherein the protozoa comprises *Cryptosporidiun parvum* and the PCR primers comprises SEQ ID NO: 1 and SEQ ID NO: 2.

13. The method according to any one of the preceding claims, wherein the sample solution comprises a water sample.

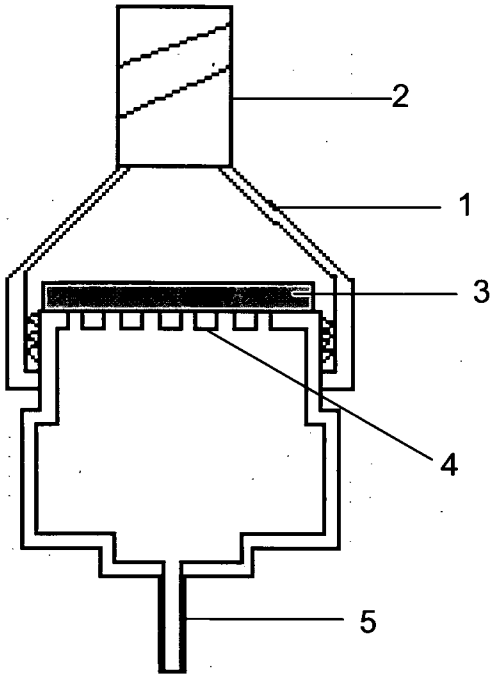


Figure 1

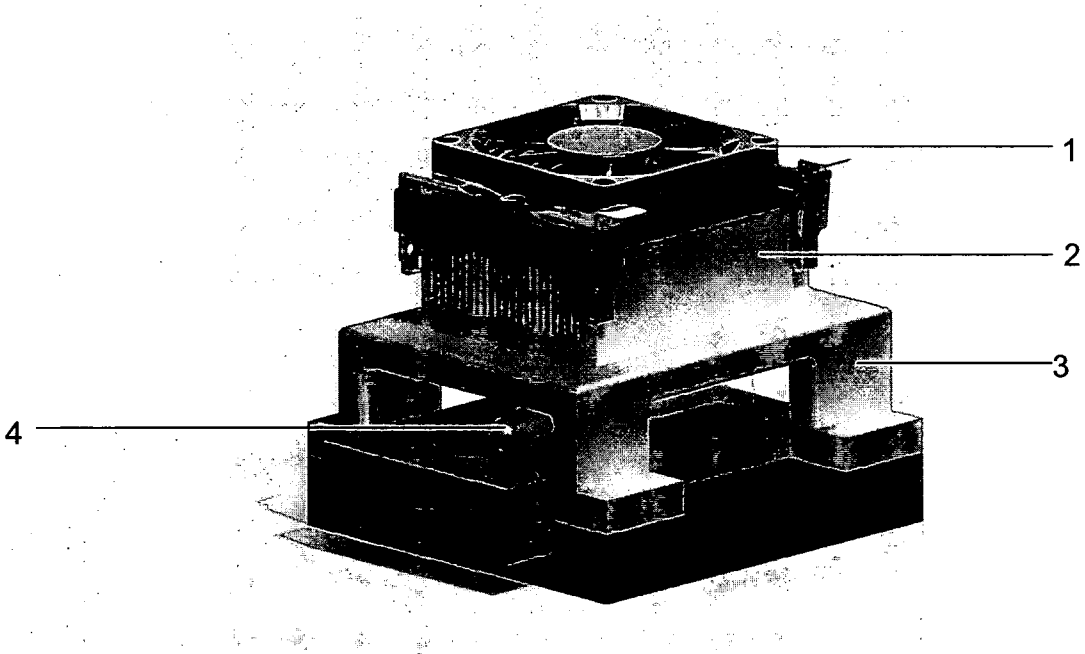
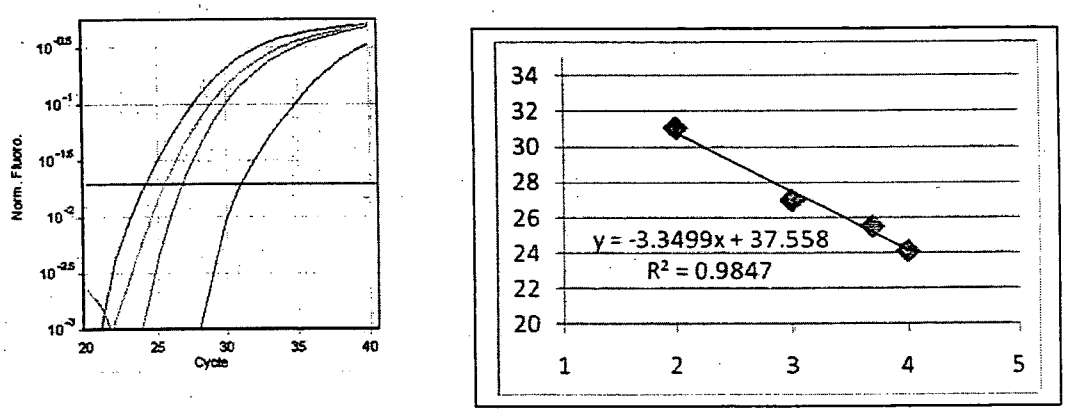


Figure 2



(a) (b)

Figure 3

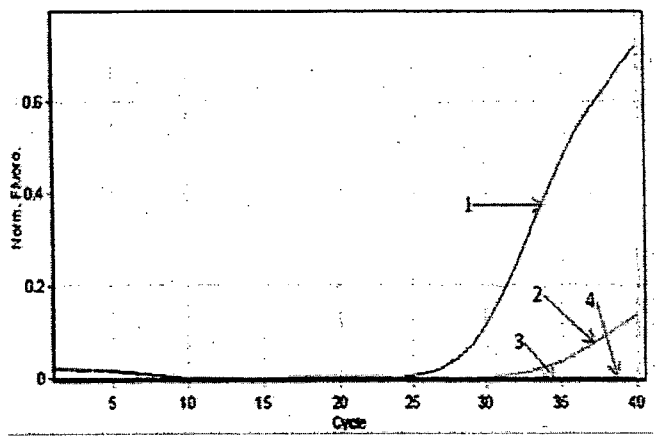


Figure 4

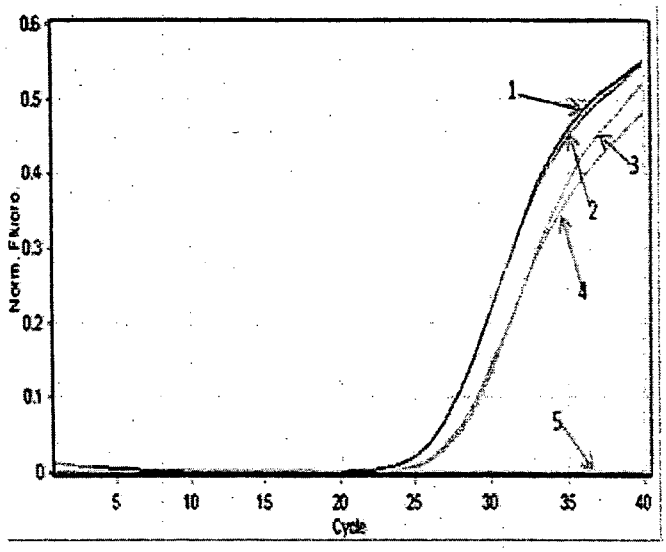


Figure 5

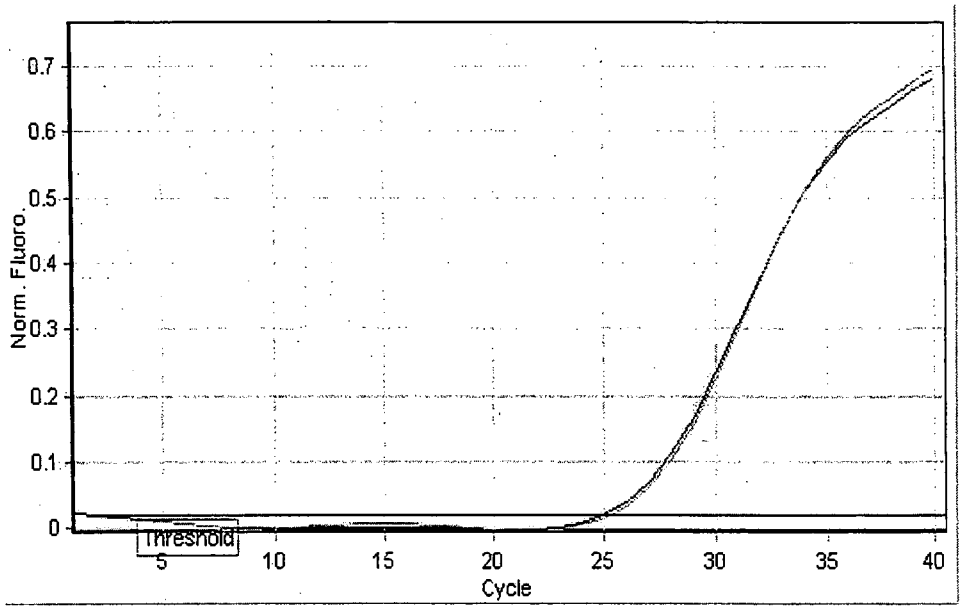


Figure 6

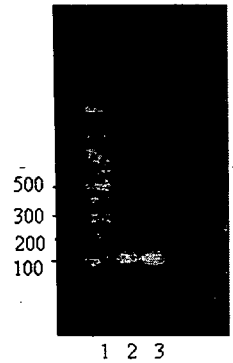


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2010/000379

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/68 (2006.01) ***C12Q 1/04*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC, WPI, MEDLINE, HCA, BIOSIS (phenanthridium, propidium monoazide, PMA, ethidium monoazide, EMA, intercalate, dye, protozoa, amoeba, ciliat, flagellate, giardia, entamoeba, sporozoa, plasmodium, apicomplexa, myxozoa, microsporidia, balantidium, cryptosporidium, euglena, leishmania, trypanosome, trophozoa, bradyzoa, tachyzoa, sarcocystis, toxoplasmo, viable, viability, dead, membrane compromised, intact, PCR, DNA, amplify, filter, filtration)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/055810 A1 (U.S. ENVIRONMENTAL AGENCY) 30 April 2009. See Abstract, Claims, paragraphs [0013] – [0014], [0024] – [0035] and [0037] – [0048].	1 – 13
Y	As above.	1 – 13
Y	BRESCIA, C.C. et al. '*Cryptosporidium* Propidium Monoazide-PCR, a Molecular Biology-Based Technique for Genotyping of Viable *Cryptosporidium* Oocysts', Applied and Environmental Microbiology, 2009 (Epub 11 Sep 2009), Vol. 75, No. 21, pages 6856 – 6863. See Abstract, pg. 6857 – 6858.	1 – 13

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2011	12 JAN 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **JAMES ALDERMAN** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 3 9935 9613

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2001/077379 A2 (MATFORSK) 18 October 2001. See Claims, Abstract, Examples 1 and 2.	1 – 13
Y	VESPER, S. et al. 'Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA)', Journal of Microbiological Methods, 2008, Vol. 72, No. 2, pages 180 – 184. See Abstract, Materials and Methods.	1 – 13
Y	US 2009/0081772 A1 (Cayre) 26 March 2009. See Abstract and Claims.	1 – 13
Y	US 2008/0213870 A1 (Cao et al.) 4 September 2008. See Abstract and Claims.	1 – 13
Y	NOCKER, A. et al. 'Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells', Journal of Microbiological Methods, 2006, Vol. 67, No. 2, pages 310 – 320. See Abstract.	7
P,X	WO 2010/110741 A1 (GONG, Haiqing et al.) 30 September 2010. See Abstract, Claims, pg. 9 line 23.	1 – 10, 13
A	NOCKER, A. et al. 'Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology', Applied and Environmental Microbiology, 2007, Vol. 73, No. 16, pages 5111 – 5117. See whole document.	
A	RUDI, K. et al. 'Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples', Applied and Environmental Microbiology, 2005, Vol. 71, No. 2, pages 1018 – 1024. See whole document.	
P,A	WO 2010/110740 A1 (GONG, Haiqing) 30 September 2010 See whole document.	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2010/000379

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2009055810	US	2009123959				
WO	0177379	AU	46712/01	CA	2405769	EP	1272664
		NO	20024879	US	2003203374	US	7262009
US	2009081772	EP	2191250	FR	2921490	FR	2921491
		WO	2009047436				
US	2008213870	NONE					
WO	2010110741	WO	2010110740				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX