Rapid method for viable Cryptosporidium parvum oocysts detection
X. N. Chu, J. Y. Hu, H. L. Guo and X. L. Tan

ABSTRACT

Cryptosporidium parvum is a waterborne coccidian protozoan parasite known to infect humans, resulting in an illness known as cryptosporidiosis. The widely used USEPA method 1622 to detect Cryptosporidium is time consuming, and unable to provide the information on oocysts viability and species. In order to develop a fast detection method for viable C. parvum oocysts, a 0.2 μm pore size hollow fiber membrane and 0.45 μm pore size disc membrane were used to filter tap water. Modified immunomagnetic separation (IMS) purification was followed, and the IMS condition was optimized to shorten the purification time. Finally, fluorescence in situ hybridization (FISH) and FITC-conjugated monoclonal antibody (FMAb) staining were combined to detect viable oocysts, and results indicated that the viable oocysts detection by FISH/FMAb can be achieved in 20 min. The total viable oocysts detection time can be shortened to less than 1.5 h without affecting oocysts recovery.

Key words | Cryptosporidium parvum, detection, filtration, FISH, viability

INTRODUCTION

Cryptosporidium parvum is a waterborne coccidian protozoan parasite known to infect humans, resulting in an illness known as cryptosporidiosis. The large cryptosporidiosis outbreak of 1993 in Milwaukee, WI, resulting in approximately 403,000 cases, raised awareness about waterborne transmission of cryptosporidiosis. LeChevallier et al. surveyed 66 water treatment plants in the Eastern United States and the Canadian province of Alberta, and found C. parvum oocysts in 87% of raw water samples (LeChevallier et al. 1994). Oocyst, as an environmentally stable form of Cryptosporidium, can be transmitted by the fecal-oral route through contaminated drinking water. Owing to its potency even at low doses, high resistance to most conventional disinfectants and no approved effective drug, C. parvum was of major concern in previous decades (Carey et al. 2004). Previous microscopic observation showed that less than 10% of oocysts found in potable water contained sporozoites, whereas approximately 33% of oocysts observed in raw water samples contained sporozoites. Hence, detecting C. parvum oocysts in conjugation with a viability assay is very important.

USEPA Methods 1622 and 1623, which are conventional methods based on immunofluorescence, are currently in use for microscopic detection of the presence of C. parvum in both treated and raw water samples (Wiedenmann et al. 1998). These methods involve four key processing steps: filtration, immunomagnetic separation (IMS) of the oocysts from the material captured, fluorescence-antibody staining of oocysts, with confirmation through vital dye staining and differential interference contrast (DIC) microscopic evaluation. However, these methods are labor intensive, time consuming, and are unable to provide information on oocyst viability and species.

To detect C. parvum from water samples, several methods have been developed. These detection methods typically contain three steps: concentration, purification and detection. The common concentrating methods include continuous calcium carbonate flocculation, cartridge filtration, centrifugal flotation, and membrane filtration (Carey et al. 2004). Centrifuge flotation needs intensive equipment. Flocculation is time consuming and is reported to reduce viability of oocysts (Shepherd & Wyn-Jones 1996).
To increase detection sensitivity, a purification step is necessary after the concentration step to remove unwanted interfering particles. Density gradient flotation including sucrose, percoll, cesium chloride or salt solution, and IMS are two methods generally used to purify oocysts from environmental debris. The IMS technique uses antibody coated magnetic beads to bind oocysts and to separate the oocysts-bead complex from the concentrated water sample by magnetic means. Some studies have reported that IMS is a superior alternative to flotation techniques (McCuin & Clancy 2003). Hsu and Huang’s report indicated that oocyst recovery was 69.3 ± 13.5% with IMS, and 29.9 ± 20.3% with flotation (Hsu & Huang 2000). Flotation methods often reduce the amount of oocysts along with interfering materials. Sample turbidity can affect oocyst recovery (Bukhari & Smith 1995). Both USEPA methods 1622 and 1623 employ IMS to purify Cryptosporidium oocysts and Giardia cysts. However, USEPA methods 1622 and 1623 cannot be used to differentiate viable and non-viable oocysts or cysts.

To detect viable oocysts, in vitro excystation combined with immunofluorescence assay (IFA), reverse transcriptase-polymerase chain reaction (RT-PCR), animal infectivity models, and fluorescence in situ hybridization (FISH) are generally used. To compare these technologies, Table 1 compares the advantages and disadvantages of each technology.

*In vitro* excystation assay can detect viable *C. parvum*, as dead oocysts do not excystate sporozoites under the appropriate conditions such as temperature, bile salts or trypsin. After excystation, IFA assay is applied. Organic fluorophores such as FITC are utilized to conjugate with anti-Cryptosporidium sp. monoclonal antibody (Mab) to label the oocysts, and the results can be detected with a microscope (Current 1987). However, an experienced observer would be needed for effective detection. Animal infectivity models usually adopt the use of mice as animal models to detect viable and infective Cryptosporidium. However, the time span taken to obtain results is quite long as it takes time for mice to react to infection by Cryptosporidium parasites. Also, it is not possible to detect low doses of *C. parvum* as no infectivity could result. The strategy for reverse transcriptase-PCR (RT-PCR) is to target viable oocysts by analyzing mRNA transcripts of a target sequence as transcription only takes place in viable cells and mRNA cannot be recovered in detectable amounts from dead organisms (Stinear et al. 1996; Wiedenmann et al. 1998; Hallier-Soulier & Guillot 2005). However, the number of viable oocysts detectable by RT-PCR was quite high; the lowest number reported was $10^3$. By contrast, fluorescence *in situ* hybridization (FISH) could detect even a single viable oocyst (Graczyk et al. 2005). The use of FISH to detect viable Cryptosporidium in environmental samples has been widely accepted. Fluorescently labeled oligonucleotide probes target intracellular 18S rRNA which is degraded after the oocysts die (Vesey et al. 1998; Smith et al. 2004). In addition, FISH assay is proved to agree with results from *in vitro* excystation assay and mouse infection assays.

The objective of this work is to develop a fast detection method for recovery, isolation and detection of total and viable *C. parvum* in treated water. The aim of this study is to shorten the detection time to the shortest without losing any oocyst recovery. Tap water from the National University of Singapore water science and technology laboratory was used for development of the method. Hollow fiber membranes and hydrophilic mixed cellulose ester membranes were used to concentrate water which was spiked with oocysts. Modified IMS was followed to purify the oocysts. Recovery was evaluated by seeded oocysts. By using these membrane filtration steps and IMS, the pre-treatment procedure before detection was supposed to be shortened without losing any recovery. After this, FISH/FMAb (FITC conjugated monoclonal antibody) was used to stain total and viable oocysts, which were studied by using spiked live oocysts.

<table>
<thead>
<tr>
<th>Detection technologies</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td><em>In vitro</em> excystation/IFA</td>
<td>Easier to operate</td>
<td>Need experienced observer; time consuming</td>
</tr>
<tr>
<td>Animal infectivity model</td>
<td>Accurate</td>
<td>Time consuming; unable to detect low doses of <em>C. parvum</em></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Fast; suitable for multiple samples</td>
<td>Detectable viable oocysts number is high</td>
</tr>
<tr>
<td>FISH</td>
<td>Fast and single viable oocyst can be detected</td>
<td>Microscope observation will be needed</td>
</tr>
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</table>

Table 1 | Comparison of viable *C. parvum* detection technologies
MATERIALS

The $10^7$ live *C. parvum* oocysts (Iowa strain) were obtained from Waterborne Inc. (New Orleans, LA). Oocysts were passaged through calves, and purified by sucrose, percoll gradients, and water washes, and stored in phosphate buffered saline (PBS) containing antibiotics and 0.01% Tween 20. Live oocysts were used in the FISH/FMAb staining step only to confirm efficiency of the staining procedure. To evaluate the recovery of the membrane filtration and IMS purification steps, dead oocysts, AccuSpike™-IR *C. parvum* oocysts, were used. AccuSpike™-IR *C. parvum* oocysts were also purchased from Waterborne and contained 100 flow cytometry sorted *C. parvum* oocysts, suspended in 0.75 mL of reagent water with 0.01% Tween 20, which had been inactivated and preserved. An anti-*C. parvum* IMS purifier kit (Dynabeads® GC-Combo IMS kit, Waterborne) was utilized to separate oocysts from other interfering particulate matter by IMS protocol. Samples were transferred to well slides and stained with FMAb. The slides were then examined by using a fluorescence microscope. To evaluate the concentration efficiency of membrane disc, the membrane was placed onto stainless steel filter housing equipment to filter 100 mL distilled water which was spiked with AccuSpike™-IR *C. parvum* oocysts (100 oocysts). After that, the membrane was immersed in a 10 mL 1× IMS buffer and sonicated using an Elmasonic S60 for 1 min. Then IMS and FMAb staining were processed for microscopic observation.

IMS purification

To shorten IMS purification time, the standard IMS protocol was modified with different initial water volumes (1, 5, and 10 mL), varied magnet bead concentrations (1×, 2×, 5×, and 10×), and a range of incubation times (10 min, 30 min and 1 h). AccuSpike™-IR *C. parvum* oocysts (100 oocysts) were spiked into different initial volumes of water. FMAb staining was followed after IMS purification. The recovery of each IMS condition was examined using microscopic observation after FMAb staining.

FISH/FMAb staining

To differentiate viable and non viable oocysts, FISH was used to detect the samples. A fluorescently labeled Cry1 probe (5' CGG TTA TCC ATG TAA GTA AAG-3') which targets the positions between 138 and 160 on the 18S rRNA and does not hybridize with oocysts of *C. baileyi* and *C. muris* was utilized (Vesey et al. 1998). $10^7$ live oocysts recovery of hollow fiber filtration and membrane disc filtration were evaluated separately as described below. 10 L tap water spiked with AccuSpike™-IR *C. parvum* oocysts (100 oocysts) was filtered through hollow fiber membrane at 2 L/min flow rate. Then 100 mL distilled water was used to back wash the membrane. To evaluate oocysts recovery of hollow fiber filtration, the 100 mL water was concentrated by centrifugation at 1,500g for 15 min, the supernatant was carefully aspirated until the volume above pellet was 5 mL. Distilled water was then added so that the pellet volume was 5% or less in 10 mL samples. An anti-*Cryptosporidium* IMS kit was utilized to separate the oocysts from other interfering particulate matter by IMS protocol. Samples were transferred to well slides and stained with FMAb. The slides were then examined by using a fluorescence microscope. To evaluate the concentration efficiency of membrane disc, the membrane was placed onto stainless steel filter housing equipment to filter 100 mL distilled water which was spiked with AccuSpike™-IR *C. parvum* oocysts (100 oocysts). After that, the membrane was immersed in a 10 mL 1× IMS buffer and sonicated using an Elmasonic S60 for 1 min. Then IMS and FMAb staining were processed for microscopic observation.

METHOD

Membrane filtration

Hollow fiber filtration combined with membrane disc filtration was used to concentrate a water sample from 10 L to 10 mL. To evaluate the oocysts recovery of each filtration step, oocysts recovery of hollow fiber filtration and membrane disc filtration were evaluated separately as described below. 10 L tap water spiked with AccuSpike™-IR *C. parvum* oocysts (100 oocysts) was filtered through hollow fiber membrane at 2 L/min flow rate. Then 100 mL distilled water was used to back wash the membrane. To evaluate oocysts recovery of hollow fiber filtration, the 100 mL water was concentrated by centrifugation at 1,500g for 15 min, the supernatant was carefully aspirated until the volume above pellet was 5 mL. Distilled water was then added so that the pellet volume was 5% or less in 10 mL samples. An anti-*Cryptosporidium* IMS kit was utilized to separate the oocysts from other interfering particulate matter by IMS protocol. Samples were transferred to well slides and stained with FMAb. The slides were then examined by using a fluorescence microscope. To evaluate the concentration efficiency of membrane disc, the membrane was placed onto stainless steel filter housing equipment to filter 100 mL distilled water which was spiked with AccuSpike™-IR *C. parvum* oocysts (100 oocysts). After that, the membrane was immersed in a 10 mL 1× IMS buffer and sonicated using an Elmasonic S60 for 1 min. Then IMS and FMAb staining were processed for microscopic observation.
C. parvum oocysts were diluted 10,000 times with PBS to a concentration of around 100 oocysts per mL. The FISH/FMAb procedure followed Vesey’s work with some modification (Vesey et al. 1998). Different fixation and permeabilization conditions before FISH were compared, and the simplest method that gave optimal fluorescence levels after FISH and minimal cluster formation (<1% clusters) was pre-treating oocysts in 1:1 ethanol/PBS for a period of 10 min (Deere et al. 1998). Hence, this pretreatment condition was chosen. 1 mL diluted oocysts were incubated in 1:1 ethanol/PBS at 80°C for 10 min to inactivate oocysts and increase oocyst wall permeability. After the pretreatment, oocysts were washed with PBS at room temperature. To undertake hybridization and immunofluorescence staining together, the washed oocysts were centrifuged at 10,000g for 2 min, and the pellets were resuspended in 100 μL of hybridization buffer with an oligonucleotide probe and MAb at 48°C for 20 min to 1 h. The concentration of the oligonucleotide probe was 1 mM and MAb was diluted at 1:1 ratio. The hybridization buffer contained 0.9 M NaCl, 20 mM Tris-HCl and 1% SDS. After incubation, the samples were centrifuged at 10,000g for 2 min. The pellets were resuspended in 100 μL of PBS. Five 20 μL samples were transferred into wells of a 8 mm well slide and a coverslip was applied. A fluorescence microscope was used to observe all the wells on the slide.

RESULTS

Membrane filtration

Hollow fiber membrane and membrane paper were utilized to concentrate C. parvum from water and the performances were compared using oocyst recovery efficiencies. In total, 100 C. parvum oocysts were dosed into 10 L tap water, and filtered by a hollow fiber membrane, followed by backwash, centrifugation, IMS, and FMAb staining. The results are shown in Table 2. The recovery of oocysts from 10 L water after hollow fiber membrane filtration, centrifugation, IMS and FMAb staining was 18.5 ± 4.9% (n = 2). Alternatively, 1 mL pre-spiked C. parvum were dosed into 100 mL distilled water, and filtered through membrane paper, followed by IMS and FMAb staining. The recovery of oocysts after membrane paper filtration, IMS and FMAb staining was 81.0 ± 4.2% (n = 2). If both hollow fiber membrane filtration and membrane vacuum filtration were combined to filter through 10 L tap water with 100 spiked oocysts, the recovery of C. parvum after filtration, IMS and FMAb was 16.8 ± 7.8% (n = 2).

The filtration recovery results indicated that membrane paper filtration could be used as a concentration method replacement for the centrifugation step, as centrifugation was not suitable to concentrate the sample in development of real time monitoring. It can also be used after hollow fiber filtration to concentrate the water sample from 100 to 10 mL or less.

IMS purification

IMS can be used to purify oocysts from environmental particles. It can also concentrate the water sample from mL to μL level. Hence, it is widely used to purify oocysts. In this study, an IMS kit used for method 1622 was utilized to study the purification time. 100 oocysts were spiked in different volumes of distilled water with different concentrations of magnet beads and buffer. This procedure was followed by incubation, bead separation, and FMAb staining. The results are shown in Table 3. With the standard procedure described in method 1622, using 10 mL initial volume, 1× magnet beads

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Volume (L)</th>
<th>Mean oocysts recovery</th>
<th>After membrane paper filtration, IMS and FMAb</th>
<th>After hollow fiber filtration, membrane filtration, IMS and FMAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 oocysts/L</td>
<td>10</td>
<td>18.5 ± 4.9%</td>
<td></td>
<td></td>
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<tr>
<td>1 oocyst/mL</td>
<td>0.1</td>
<td></td>
<td></td>
<td>81.0 ± 4.2%</td>
</tr>
<tr>
<td>10 oocysts/L</td>
<td>10</td>
<td></td>
<td></td>
<td>16.5 ± 7.8%</td>
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and 1 h incubation time, 91.5% oocysts can be detected after IMS. The IMS procedure took at least 1.5 hours, of which the incubation time was 1 h for antibodies to bind with oocysts. The results in Table 3 indicate that the IMS step can be shortened from 60 to 30 min. The optimal IMS condition was Assay 3, with five times more beads and 1 mL of reaction volume, under which condition, comparable oocysts recovery to standard method, 89.5%, can be achieved.

**FISH/FMAb staining**

It has been reported that FISH can be used to detect the viable *Cryptosporidium* in environmental samples. The possible mechanism is that fluorescently labeled oligonucleotide probes target intracellular 18S rRNA, while rRNA only occurs in live oocysts. FISH may also give an indication of oocyst species (*Vesey et al. 1998*). The standard FISH procedure takes more than 2.5 h. FMAb staining takes another 40 min. In this paper, FISH and FMAb staining were done simultaneously to decrease the incubation time. The results indicate that FISH and FMAb staining time can be shortened from 1 h to 20 min as shown in Figure 1 to detect total and viable *C. parvum* oocysts. After 20 min incubation, both FISH and FMAb staining can give fluorescence signals as strong as 1 h incubation. In Figure 1, FMAb staining gave a green signal while FISH staining gave red signal. FMAb stained total oocysts, while FISH stained viable oocysts only. Figure 1 indicates that although the oocysts after 20 min incubation were not as legible as the ones after 1 h incubation, the oocysts’ shapes and walls were obvious, which can be used to differentiate interference particles. An incubation time shorter than 20 min was also examined, but the fluorescence signal was not strong enough for observation. Hence, a 20 min FISH/FMAb reaction was the shortest incubation time for quick oocyst detection from treated water samples with less turbidity. In the next stage, quantum dots (QDs) may be introduced to replace organic fluorescence in the oligonucleotide probe or FMAb, as QDs are reported to have the advantages of brighter signal, greater photostability, and minimal interference from natural autofluorescent particles (*Lee et al. 2004*), which makes it possible for them to be used for fast oocyst detection from environmental water concentrates containing autofluorescent algae and mineral particles.

### DISCUSSION

In Feng et al.’s (2003) study in 2003, around 2,000 oocysts were spiked into 10 L tap water to study oocyst recovery at each step of method 1622. An Envirocheck capsule filter was used. They found that the levels of oocyst recovery following different treatments were 98.5% after FMAb staining, 95.1% after IMS and FMAb staining, and 14.4% after filtration, IMS and FMAb staining. These results indicated that filtration was the key step that affects oocyst recovery. This study also showed that the filtration step limited the oocyst recovery. The efficiency of five membrane filters for recovery of *Cryptosporidium* oocysts was evaluated.
by Wohlsen and his colleagues in 2004. One hundred oocysts were spiked into 10 L distilled water and raw water and were filtered through filters including Pall Life Science’s Envirocheck standard filters (EC), Envirocheck high volume membrane filters (EC-HV), Millipore flatbed membrane filter, Sartorius flatbed membrane filter (SMF) and FilterMax depth filter (FM). For distilled water, EC-HV was found to be the best filter to recover oocysts with recovery of 53% followed by FM with recovery of 28.2%. However, filter elution needs 60 min for EC-HV, longer than the others. FM needs one more step: vacuum sample concentration. The recovery range of other filters was from 0.2 to 18.4% (Wohlsen et al. 2004). Recovery of filtration of this study was comparable to these results. Shepherd and Wyn-Jones compared membrane filtration, flocculation and cartridge filtration for recovery of Cryptosporidium from tap and river water samples. Flocculation was proved to be the method with the highest oocyst recovery; however, flocc development needs 4 h to overnight, which was not suitable for fast detection. They found Vokes cartridge filtration had a mean recovery of 11.2 and 9.4% from tap and river water samples, respectively. Their study of Cryptosporidium recovery by membrane using a Versapor membrane, cellulose acetate membrane, and polycarbonate membrane showed that 1.2 μm cellulose acetate membrane was optimal, with recovery of 39.7% from tap water, which was believed to be due to the smooth surface of the cellulose acetate membrane. The recovery of 0.6 μm polyethylsulfone membrane was 24.0% from tap water. Even with smaller pore size and smooth surface, the recovery of a 0.6 μm polyethylsulfone membrane was lower than expected because of compaction of particulates onto the surface. Shepherd and Wyn-Jones suggested the reason for low recovery was that either the membrane pores were too small or the membrane binding capacity to oocysts had irreversibly attached many oocysts (Shepherd & Wyn-Jones 1996). Also in their study, sample elution after filtration was achieved by scraping the membrane with a smooth edged plasticine molder, which was also tried for the membrane disc in our current study. But scraping may damage the membrane surface. Some scraps of membrane were eluted into the elution, which affected downstream steps and microscope observation. In this study, sonication was utilized to elute oocysts from the membrane disc in minutes with recovery of 81%, which was not reported in the oocysts study. In addition, sonication was used to separate bacteria cells from particles before FISH without affecting viability results (Lam & Cowen 2004). This study proved that sonication can be a good elution solution after membrane filtration. Sonication conditions need to be optimized in future studies to increase recovery of filtration without affecting viability of oocysts.

Although the recovery of hollow fiber filtration was comparable with Feng et al.’s results, and some membrane filters’ recovery results in Wohlsen et al.’s study, recovery of hollow fiber membranes was not satisfied. Too small pore size was considered as one of the reasons for the low recovery. Additionally, another reason was suspected to be the high binding capacity of the hollow fiber membrane surface to oocysts, which would affect the efficiency of backwashing. In future, membranes with bigger pore size and smoother surface could be employed to increase recovery and decrease filtration time. The utilized hollow fiber membrane was a special model with fiber exposed to water directly. If a membrane with casing was used, gas can be added for the backwash to decrease the final volume of the concentrate.

IMS results suggested that the more magnet beads, the higher recovery under the same incubation time. Smaller reaction volume and more beads can enhance IMS recovery. This is due to the increase of opportunities of oocysts to contact with antibodies on the beads. In assays 2 and 3, samples were incubated for 50 min in 1 mL reaction solution. Their recoveries were 68.5% for assay 2 using 2.5× beads and 89.5% for assay 3 using 5× beads. This indicated that the more beads in the reaction solution, the higher the recovery, as opportunities of antibodies on the beads contacting the oocysts were increased. However, when the incubation time was as short as 10 min, increasing beads concentration did not improve recovery, as shown in results of assays 4 and 5. Although beads concentration was increased two times, from 5× to 10×, recovery was increased from 12.5 to 16% with only a 3.5% improvement, comparing with greater improvement of recovery from 68.5 to 89.5% with two times beads increasing in assay 2 and 3. This suggested that the binding time between antibodies on the beads and oocysts needs to be long enough after the antibodies receive the oocysts. 30 min was suspected to be the minimal incubation time for antibodies to bind with oocysts. Thus, currently 30 min was believed to be the shortest incubation
time of IMS without affecting recovery. In Feng et al.’s (2003) study of method 1622, 95% of Cryptosporidium parvum was recovered from a concentrated tap water sample when IMS was used to purify samples. In Hu et al.’s (2004) study on method 1623, around 200 C. parvum and 200 Giardia were spiked into 10 mL reagent water. 92% of oocysts were recovered when IMS and IFA were used to purify and stain oocysts. As the recovery result was close to levels determined using method 1622, the authors suggested that IMS time can be shortened by half, although the cost of detection would be increased as more IMS beads were used. This study showed that IMS time can be shortened by half, although the cost of detection would be increased as more IMS beads were used.

**CONCLUSIONS**

In this study, a fast viable C. parvum detection method was developed. A 5 min hollow fiber filtration and 2 min membrane filtration were combined to concentrate water, followed by 30 min fast IMS to purify oocysts from the concentrated water sample. After this, FISH and FMAb were combined to stain oocysts to identify viable and non-viable oocysts, which need 34 min including centrifuge. The fast detection procedure needs less than 1.5 h totally, which has not been reported before. However, this method was evaluated step by step. Thus the overall procedure from 10 L water sample to microscope examination needs to be evaluated in a future study. Double filtration was able to concentrate a water sample with acceptable recovery. Recovery can be enhanced if smoother hollow fiber or a membrane with bigger pore size are employed. The optimized IMS condition speeded up the IMS step while keeping the same level of oocyst recovery. Oocyst detection by FISH/FMAb was proven to work for a water sample without autofluorescent particles when incubation time was shortened. QD was suggested to replace organic fluorescence to give a brighter signal and minimal interference from natural autofluorescent particles.

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**REFERENCES**


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