Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

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Deliverable D6.3

Procedures to extract high quality nucleic acids from water concentrates for pathogen detection.

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1. Introduction.

The use of molecular assays for detection of pathogens has increased following the rapid development of sequencing method which has provided information on microbial genomes. Molecular assays based on PCR or qPCR has been developed to detect and quantify pathogens in a much shorter time than required for conventional culture or microscopy methods. Assays may be designed to detect a wide range of microorganisms, a genus, a specific microorganism or a genetic marker with a high sensitivity. However, an important part of any protocol is the quality of extracted nucleic acids, which must be effectively purified from the samples and cells as this is vital for any successful detection by PCR.

Many kits are commercially available for extraction of nucleic acids from matrices like bacterial and eukaryotic cell cultures, blood, feces or soil. For water samples, much fewer kits exist and these are usually not applicable to water concentrates but made for direct extraction from small volumes or water or from flat membranes after filtration. Surface waters often contain a lot of different compounds such as plant matter, soil, sediment and various other organic and inorganic substances. These include substances that may inhibit the PCR, for example phenols, fulvic and humic acids (Kreader et al 1996, Katcher and Schwartz 1994, Opel et al 2010). Also finished waters may contain inhibitory compounds such as monovalent and divalent cations (Abu Al-Soud and Rådström 1998, 2001). These inhibitors hamper molecular detection and may result in false negative results or decrease the sensitivity, i.e. detect a much lower number of genomes from the pathogens actually present in the water. Much is gained if microorganisms can be separated from inhibitory compounds before nucleic acids are extracted but they can also be removed during extraction or after extraction, e.g. using spin columns specifically designed to remove inhibitory compounds.

The chosen extraction technique will depend on whether DNA, RNA or both are wanted. RNA is less stable than DNA, and can be degraded unless stored properly and may also require addition of stabilizing agents. Extraction can be separated into two different approaches 1) chemical lysis using enzymes such as Proteinase K, detergents (SDS, Tween) or chaotropic salts (usually guanidinium thiocyanate) and 2) mechanical lysis such as boiling, freeze-thawing or bead beating. For hard to lyse structures, the two are often combined. Mechanical lysis may result in shearing of the nucleic acids which might be a problem if long nucleic acid fragments are needed for detection but this is not usually the case for (RT)-qPCR based assays. Vegetative bacteria and viruses are usually fairly easy to lyse while bacterial endospores and protozoan (oo)cysts are more difficult. After lysis, the nucleic acid needs to be purified and concentrated, usually by binding to silica immobilized on columns or magnetic beads, which are extensively washed before the nucleic acids are eluted and collected.

2. Aim

The work has been aimed at developing protocols for nucleic acid extraction from Gram negative and positive bacteria, enveloped and non-enveloped viruses and Cryptosporidium oocysts from tap and surface water concentrates. As Cryptosporidium oocysts require much harder treatment to ensure complete lysis, focus has been on methods that can be used for 1) all three types of organisms simultaneously (bacteria, viruses and protozoan (oo)cysts) and 2) bacteria and viruses only. The last method requires fewer reagents, less equipment and hands-on time for use when detection of parasites is not of interest.

3. Method for "all-in-one" Nucleic Acid Extraction

The aim was to provide a user friendly protocol using as little equipment as possible, ideally only what is normally present in a water laboratory, or at least not very expensive.

During the method development, the following model organisms were used, the Gram negative bacteria *Campylobacter jejuni* and *Salmonella enterica* subsp. *enterica* serovar enteritidis, the undeveloped viruses murine norovirus and MS2 phages and *Cryptosporidium parvum* oocysts. The method has also been shown to work for Mengovirus and *Listeria monocytogenes* (model for Gram positive bacteria).

For practical reasons, mainly "in house" qPCR assays in combination with inhibitor tolerant (RT)-qPCR master mixes have been used for detection (Table 1). The Cq-values obtained from extracts in the different protocol has been used to compare the efficiency of the different protocols, i.e. a lower Cq-value means that a method has released more DNA/RNA than one that gives a higher Cq-value.

Table 1. Primers and probes used for qPCR.

Primers	Gene	Primers and probe	Master mix	Reference
C. jejuni	*hip*O	HipOF 5'-AATGCACAAATTTGCCTTATAAAAGC-3' HipOR 5'-TNCCATTAAAATTCTGACTTGCTAAATA-3' HipOP HEX-ACATACTACTTCTTTATTGCTTG-CCAATCACAGAATCATCAGAATCGACTGGTATC-BHQ1	PerfeCTa® qPCR Toughmix	Toplak et al 2012
L. monocyte-genes	*hly*A	HlyF 5'-TGCAAGTCCTAAGACGCCA-3' HlyR 5'-CACTGCATCTCCGTGGTATACTAA-3' HlyP FAM-CGATTTCATCCGCGTGTTTCTTTTCG-BHQ1	PerfeCTa® qPCR Toughmix	Nogva et al 2000
***S.* enteritidis**	*inv*A	SalF 5´-TCGTCATTCCATCTACC-3´ SalR 5'-AAAACGTTGAAAAACTAGAGGA-3' SalP FAM-TCTGGTTGATTTCCTGATCGCA-BHQ1	PerfeCTa® qPCR Toughmix	Hoorfar et al 2000
***E. coli* O157**	*rfb*E	O157 F 5´-TTTCACACTTATTGGATGGTCTCAA-3' O157 R 5'- CGATGAGTTTATCTGCAAGGTGAT-3' O157 P FAM-AGGACCGCAGAGGAAAGAGAGGAATTAAGG - TAMRA	PerfeCTa® qPCR Toughmix	Perelle et al 2004
MS2 phage		MS2 F 5'-TGCCATTTTTAATGTCTTTAG-3' MS2R 5'-TGGAATTCCGGCTACCTAC-3' MS2P FAM- AGACGCTACCATGGCTAT-BHQ1	TaqMan® Fast Virus 1.Step Master Mix	Hill et al 2007
Murine norovirus		MNV-F 5´- TTGGGAACATGGAGGTTCAR - 3´ MNV-R 5´- GGRAAATAGGGTGGTACAAGG – 3´ MNV-P FAM – CCA*CCT*TGC*CAG*CAG*T-BHQ1 *LNA	TaqMan® Fast Virus 1.Step Master Mix	Swedish National Veterinary Institute Unpublished
Crypto-sporidium sp*		CrF 5´- CGCTTCTCTAGCCTTTCATGA- 3´ CrR´ 5´- CTTCACGTGTGTTTGCCAAT – 3´ CrTM FAM - CCAATCACAGAATCATCAGAATCGACTGGTATC- BHQ1 PCR protocol developed and provided by Chalmers et al (AQ-project), modified here with different fluorophore and master mix.	PerfeCTa® qPCR Toughmix PerfeCTa® qPCR Toughmix	Fontaine and Guillot, 2002 Chalmers unpublished

* Two different qPCRs were used for Cryptosporidium.

3.1. Commercially available kits for nucleic acids from soil

As a kit containing all reagents required for nucleic acid extraction would be user friendly, three different commercially available kits designed for extraction of DNA from soil samples, 1) FastDNA Spin kit for soil (MP Biomedicals), 2) NucleoSpin kit for soil (Macherey-Nagel) and 3) PowerLyzerPowersoil DNA isolation kit (Mo-Bio) were tested. Polyethylene glycol (PEG)/NaCl-precipitated Dead End Ultra Filtration (DEUF)-concentrate (from finished water) was seeded with all three organism groups with the aim to isolate both DNA and RNA. All three kits are based on bead beating using different combinations of beads in lysis buffers followed by purification on spin columns. The protocols provided by the manufacturers were followed except that a FastPrep-instrument (MP Biomedicals) at setting 6 was used with all kits. Only bacterial DNA was detected by qPCR which indicated that *C. parvum* oocysts were not lysed nor was viral RNA bound to the columns. Therefore, these kits were not tested further.

3.2. Ultra Turrax

The ULTRA-TURRAX® Tube Drive Workstation (IKA Laboratory Equipment) was tested as a cheaper alternative to a bead beating instrument in combination with a lysis buffer made according to a protocol provided by Vincent Hill (personal communication), recently published in Hill et al 2015 and that is now marketed under the name UNEX buffer (Microbiologics). The UNEX buffer is used at ratio 1:1 (sample:lysis buffer) compared to for example the NucliSense lysis buffer which is used in a ration 1:3. This is important as the volume of the bead beating tube and instrument limits the volume that can be processed. In combination with the ULTRA-TURRAX, 2 ml DEUF-concentrate could be processed compared to only 500-700 µl in the commercial kits. Four different combinations of beads were tested:

1. 10 large stainless steel beads provided with the instrument
2. 10 large stainless steel beads and 1.7 g small stainless steel beads (0.2 mm)
3. 500 mg of small stainless steel beads and 2.5 g zirconium oxide beads (0.5 mm)
4. 10 large stainless steel beads and 2.5 g zirconium oxide beads (0.5 mm)

The tubes were attached to the ULTRA-TURRAX instrument and run at 4000 RPM for 2 x 30 sec. Nucleic acids were purified using Magnetic beads, NucliSENS Magnetic Extraction Reagents and the miniMAG-instrument (BIOMÉRIEUX), or HiBind RNA-columns (Omega Biotek Store) which both binds DNA as well as RNA. Again, bacteria could be detected but not *C. parvum* suggesting that oocysts were not lysed. Very low amounts of viral RNA were detected with somewhat better results for nucleic acid extracted with magnetic beads than columns.

3.3. UNEX buffer with different combinations of beads

The paper published by Hill et al 2015 and the properties of the UNEX-buffer encouraged further work with this buffer. Portions of 700 µl seeded DEUF-concentrate were mixed with 600 µl UNEX buffer and 100 µl proteinase K ((>600 mAU/mL, Qiagen) and incubated at 37 °C for 30 minutes. After addition of different combinations of beads (see below) the samples were run at setting 6.0 in a FastPrep instrument. Nucleic acids were extracted on beads and on columns.

1. Silica, ceramic and one large glass bead from the FastDNA kit for soil
2. Ceramic beads from the NucleoSPIN kit
3. Glass beads from the PowerLyser Powersoil kit

4. Glass beads from the PowerLyser Powersoil kit with addition approximately 1.25 g zirconia beads (0.7 mm)
5. Glass beads from the PowerLyser Powersoil kit with addition of approximately 0.85 g stainless steel beads (0.2 mm)
6. Approximately 1.25 g zirconia beads (0.7 mm) and 0.85 g stainless steel beads (0.2 mm)

In this experiment, the combination of glass and zirconia beads and extraction using magnetic beads (No. 4 listed above) showed the best overall results, i.e. detection of DNA/RNA from all added organisms, even though the results for several combinations were fairly similar. Our final protocol (see Appendix 1) is also based on 1) the experience that spin columns are prone to clogging when PEG/NaCl-precipitates from turbid surface waters are processed and 2) that larger volumes of the secondary concentrates can be processed when using magnetic beads than spin columns.

3.4. Optimization of the protocol

Increasing the proteinase K incubation temperature to 56 °C and period to 1 h before bead beating, further improved the lysis of *C. parvum* oocysts. However, the proteinase K enzyme is relatively expensive and the method requires a bead beating instrument able to run at a high speed. Therefore, attempts were made to decrease the amount of proteinase K and eliminate or at least enable the use of a less expensive bead beating instrument.

First instrument settings 4.0, 4.5, 5.0, 5.5 and 6.0 instruments were compared and the results showed that settings 5.0, 5.5 and 6.0 gave very similar results, at setting 4.5 the Cq-value increased from 30.4 to 31.5 and at 4.0 no *C. parvum* DNA was detected. Neither was *C. parvum* DNA detected when the bead beating instrument was replaced by a standard vortex.

Next, the use of 100 µl, 75 µl and 50 µl Proteinase K ((>600 mAU/mL, Qiagen) in combination with 600 µl, 625 µl and 650 µl lysis buffer was investigated. Decreasing the amount of proteinase K from 100 µl to 50 µl had no effect on the detection of *C. parvum* DNA, i.e. Cq-values were similar. In the final protocol, 50 µl proteinase K and a setting of a minimum of 5.0 is recommended.

The volume of the concentrate that can be extracted is limited to 700 µl per tube. An attempt was made to increase the volume by using a Mini-Beadbeater 24 (Biospec Products) with 7 ml vial. However, this instrument were not able to run at a speed high enough to lyse Cryptosporidium (oo)cysts.

3.5. Inhibitor removal

In a project preceding Aquavalens, the *OneStep*™ PCR inhibitor Removal Kit (ZYMO Research) had been shown to improve RT-qPCR detection of viruses in surface waters while the effect for tap water was small, and sometimes even negative. Therefore, further purification of the nucleic acid on *OneStep*™ columns was included in the protocol for surface waters but not recommended for finished water.

4. Method for bacteria and viruses

To reduce the effect of PCR inhibitors it is necessary to extract nucleic acids using a method that yields clean DNA and RNA preparations. As can be seen in the Aquavalens Deliverable 6.2, successful application of e.g. the NucliSENS reagents and miniMAG apparatus from BioMerieux was obtained for both the Gram negative bacteria, *Campylobacter jejuni* and *Salmonella typhimurium*, the Gram positive bacteria, *Listeria monocytogenes,* as well as the enveloped virus, porcine transmissible gastroenteritis virus, TGEV (a model of human coronaviruses such as MERS and SARS), and non-enveloped viruses, norovirus GI and GII, murine norovirus (MNV), mengovirus and human adenovirus (HAdV), concentrated from drinking water and/or surface water. The NucliSENS miniMAG method is based on disruption of virus capsids and bacterial membranes with the chaotropic reagents, guanidine thiocyanate, followed by adsorption of DNA and RNA to magnetic silica beads to assist subsequent purification through several washing stages. Purified nucleic acids are then released from the beads into a buffer. In the studies described in the Deliverable D6.2, we also observed an overall difference in the quality of extracts purified by the NucliSENS reagents from eluates concentrated by glass wool filtration, with regard to inhibitor removal for successful detection of RNA and DNA genomes by RT-qPCR or qPCR, respectively. It was therefore concluded, that there can be inhibitors present in the extracts which is more crucial for successful amplification by RT-qPCR than by qPCR.

In a parallel project, EU FP7 MetaWater, which co-finance Aquavalens, DTU Food conducted a joint study, investigating the influence of the 1st concentration method from dirty water samples (here sewage) to obtain good quality nucleic acids with the application of different extraction methods. A summary of this study is described below.

The efficiency of methods to extract nucleic acids from viruses depends on the method used for the prior viral concentration.

An aim of the study was to evaluate the efficiency of different nucleic acid extraction methods to purify satisfactory clean viral RNA and DNA from eluates obtained using different concentration methods, to subsequently be quantified by RT-qPCR or qPCR, respectively. We wanted to determine if we could find higher quantities of viruses with methods that could process higher sample volume.

Thus, four different methods commonly used for viral concentration were combined with four different nucleic acid extraction methods (see table 2) to concentrate viral particles and then extract viral genomes from raw sewage samples. Each concentration method was applied on a different sample volume. For the flocculation methods; polyethylene glycol (PEG) and Skim milk flocculation (SMF), the chosen sample volumes, 0.2 and 10 liter, respectively, were based on the literature (van Hauen 2014; Calgua et al., 2008). The sample volumes chosen for the two other filtration methods; monolithic affinity filtration (MAF) and Glass wool, were chosen based on the maximum volume that could be filtered without clogging the filters.

Table 2: Tested method combinations for viral concentration and viral genome (RNA and DNA) extraction. Nucleic acids were extracted according to the protocols provided by the manufacturers.

Microbe Concentration Methods	Nucleic acid Extraction Methods
1. PEG precipitation (0.2 L)	1. NucleoSpin RNA XS
2. Skimmed milk flocculation (10 L)	2. Qiagen –viral RNA Mini Kit
3. MAF columns (1 L)	3. NucliSENS miniMAG Biomerieux
4. Glass wool filtration (4 L)	4. MoBio – powerviral Environmental RNA/DNA

A portion of 120 L of raw sewage collected at the waste water treatment plant were mixed thoroughly in 5 minutes with $2.09x10^{10}$ RT-PCR units of MNV and $2.56x10^{11}$ genome copies of HAdV. The volumes shown in Table 2 were then sampled as three biological replicates together with negative control samples (sterile molecular grade water) to be processed by the use of each of the four concentration methods. The virus concentrates obtained from each concentration method were then divided to be extracted with each of the four extraction methods. All samples were analyzed by (RT)-qPCR to evaluate the detection rates of the two spiked viruses.

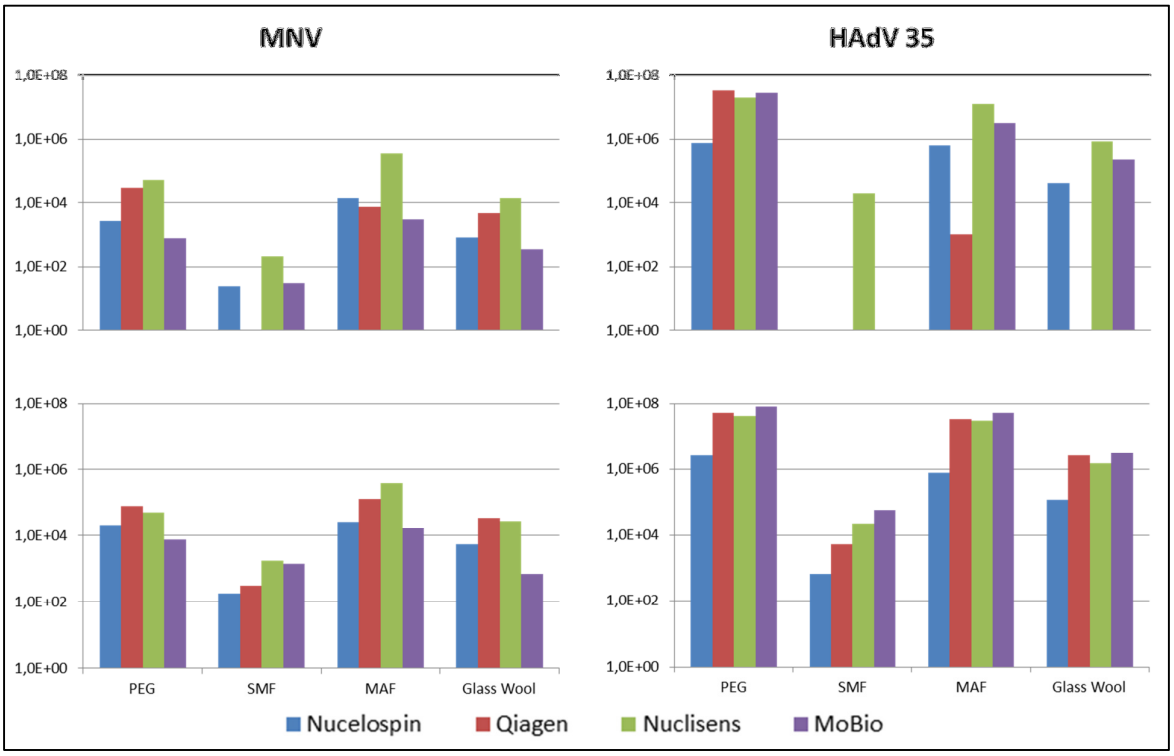

Figure 1. Detection rates of spiked murine norovirus (MNV) and human adenovirus 35 (HAdV) measured in genome copies or RT-PCR U, respectively, and calculated per L of sewage sample. Detection was carried out on undiluted (top figures) and 10-fold diluted (bottom figures) nucleic acid extracts. The bars indicate the variation in recoveries obtained by use of different extraction methods on extracts from different concentration methods.

The results are shown in figure 1 and indicate that the combination of concentration and extraction method chosen is important to obtain the most efficient quantitative recovery of the RNA and DNA genomes from the spiked viruses. Extraction by the NucliSENS kit allowed detection of both viruses and with the overall highest detection rates in undiluted extracts regardless the priory applied concentration method. Moreover, as can be seen by comparing detection rates for each virus in undiluted and diluted RNA (top and bottom figures), only minor – if any at all - inhibition was observed when testing extracts obtained from the NucliSENS kit combined with any of the four concentration methods.

In contrast, using the Qiagen kit, none of the viruses could be detected in undiluted extracts when combined with the skimmed milk flocculation or the glass wool filtration concentration methods. However, testing tenfold diluted extracts, allowed quantifiable results for both the RNA and DNA viruses in extracts obtained from all combinations of concentration and extraction methods (figure 1 bottom figures). This in spite of the names of some of the extraction kits, indicating only RNA extraction, NucleoSpin RNA XS and Qiagen viral RNA Mini Kit.

The two concentration methods, skimmed milk flocculation and glass wool filtration, that can process the highest volumes of sewage, 10 L and 4 L, respectively, resulted in the lowest detection rates of both viruses regardless the use of combined extraction kit. This is probably due to a higher concentration of inhibitory factors in these samples since they have processed a larger sample volume.

Therefore, among the four tested extraction kits applied on concentrates of different volumes of sewage processed by different concentration methods, we conclude that the NucliSENS miniMAG nucleic acid purification system from Biomerieux is the most efficient to recover both the RNA and DNA from the spiked viruses.

This study is a part of a larger study with the aim to evaluate methods for the concentration and extraction of viruses from wastewater in the context of metagenomic sequencing, which is currently in prep for publication (Hjelmsø in prep).

5. Conclusions

The use of equipment not normally available at water laboratories, such as a bead beating instrument and easyMag or MiniMag instrument, or similar, could not be avoided. These or similar instruments need to be available for the work in cluster 3.

From the work conducted in WP6, the partners conclude that the NucliSENS nucleic acid purification system from Biomerieux should be used for extraction of RNA and DNA from viruses and bacteria. This nucleic acid extraction of bacterial and viral genomes can be fully automatically processed if an easyMAG machine is available. The more manual miniMAG is also easy to use even for an inexperienced user. The bead beating protocol, however, requires more hands-on work.

Nucleic acid extraction from parasites require a more concentrated lysis buffer, UNEX buffer (see comment below), and proteinase K treatment followed by bead beating and purification using the Biomerieux reagents and equipment. This protocol can also be used for bacteria and viruses.

Most likely, all nucleic acid extracts, from for example turbid or strongly coloured surface waters, will not be completely free of inhibitory compounds. This can be solved by using inhibition tolerant qPCR master mixes (such as the PerfeCTa® qPCR Toughmix or similar) or by addition of PCR facilitators such as BSA or polyvinylpyrrolidone (PVP) for the RT-(q)PCR.

Comment: During the last year (Summer 2015-2016) we have experienced problems with the commercially available UNEX-buffer with positive signals in the negative extraction controls when running PCRs for Salmonella but not for other bacteria or Cryptosporidium. The manufacturer has now confirmed that the two batches of lysis buffers used indeed were contaminated with Salmonella DNA and that this problem now has been solved. Initial experiments at NFA were performed with a homemade version of this buffer with which the Salmonella PCRs worked as expected. This clearly shows the need to test all reagents, such as buffers and mastermixes before use, as well as include relevant negative controls.

6. References

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7. Implications of the results of Deliverable 6.3

Implications of the results of Deliverable Report 6.3

Implications of the results for the Work Package (WP 6)

The work in WP6 is more or less finished. Method 1 has been used in the filtration ring test carried out during the Spring 2016. Method two is used by DTU, NFA and UB for analysis of virus in water.

Implications of the results for this Cluster 3

Methods for extractions of nucleic acids from water concentrates are ready to use for the field studies in Cluster 3. Two protocols are provided; one for all three organism groups and one that is easier to perform and requires less equipment for samples were only viruses and bacteria are studied.

Implications of the results for the whole project

The same as the implications of the results for the Work Package (WP 6) (see above).

Indicate key external stakeholders interested in the results of Deliverable Report 6.3

D6.2 will be of value for stakeholders such as large water or small water or food suppliers as well providers of tools for molecular detection if the tools are shown to work well and thus can be recommended to be used with the protocols provided here.

Which internal partners should your deliverable be sent to?

Partners in Cluster 2 and 3

Appendix 1

Protocol for nucleic acid extraction from bacteria, viruses and parasites

Material

Equipment

Bead beating instrument (e.g. Fast-Prep, MP BioMedical)

Magnetic extraction instrument capable of collecting magnetic silica beads and of mixing magnetic silica beads in solution (e.g. NucliSENS®miniMAG)

Thermoshaker, up to 60 °C and mixing frequency 1400 rpm

Magnetic tube rack for 1.5 ml tubes

Centrifuge for the lysis buffer tubes used (2 ml or 15 ml tubes)

Micropipettes with variable settings from 10-1000 µl

Vortex

Supplies
UNEX lysis buffer (Microbiologics®)
Proteinase K (>600 mAU/mL, e.g. Qiagen 19133)
Glass beads (0.1 mm) (e.g. Next Advance)
Bead beating tube, 2 ml (e.g. BioSpec Products)
Zirconia beads (0.5-0.7 mm) (e.g. Next Avdance or BioSpec Products)
NucliSENS Magnetic Extraction Reagents (BIOMÉRIEUX)
OneStep Inihibitor Removal Kit (Zymo Research) for surface waters
Microtubes, 1.5 ml
Sterile aerosol resistant pipette tips

Procedure

Extract all samples simultaneously while including a positive and a negative extraction control:
• One positive control containing e.g. Mengovirus, the bacterium and parasite you want to detect, if possible, in a total volume of 1 ml.
• One negative control containing 1ml PBS.

If the concentrate volume is large, several replicates can be processed in parallel and pooled in a 15 ml Falcon tube before the magnetic beads are added.

Transfer 700 µl concentrate to a 1.5 ml microtube

Add 650 µl Unex lysis buffer and 50 µl Proteinase K. Mix by vortexing.

Incubate at 56 °C for 1 hour, preferably in a thermoshaker.

Spin for a few seconds at 10 000 x g and transfer the entire content to a bead beating tube containing 0.5 g of 0.1 mm glass beads and 0.5 g of 0.7mm zirkonia beads.

Vortex vigorously for 15 sec.

Run for 2 x 30 seconds in a FastPrep instrument at minimum of setting 4.5 but preferably at 6 (for other beadbeating instrument, choose a similar setting)

Spin for 30 seconds at 10 000xg.

Transfer the supernatant to a 2 ml Eppendorf tube. If several replicates have been made, they should be pooled in a 15 ml Falcon tube.

Add 50 µl magnetic beads from the NucliSENS Magentic Extraction Reagent Kit and follow the instructions provided by BIOMÉRIEUX.

NB! For surface water or other waters that may contain PCR inhibitors, run the NA extract through an *OneStep*™ Inhibitor Removal column, following the instructions provided by the manufacturer.

Appendix 2

Protocol for nucleic acid extraction from bacteria and viruses

This is the same protocol as is in the manual provided by bioMérieux

Material

Equipment

NucliSENS® easyMag® or MiniMag®| bioMérieux, Manufactures Protocol =CEN ISO

Supplies

NucliSENS® Lysis Buffer| bioMérieux
NucliSENS®Magnetic Extraction Reagents | bioMérieux
Thermoshaker, up to 60 °C and mixing frequency 1400 rpm
Magnetic tube rack for 1.5 ml tubes
Centrifuge for the lysis buffer tubes used (15 ml tubes)
Micropipettes with variable settings from 10-1000 µl
Vortex
Microtubes, 1.5 ml
Sterile aerosol resistant pipette tips

Procedure

Note: Equilibrate reagents and beads to room temperature by place at RT 10 min before use.

Extract all samples simultaneously while including a positive and a negative extraction control:
• One positive control (rna+) containing e.g. Mengovirus and the bacterium you want to detect in a total volume of 1 ml.
• One negative control (rna-) containing 1ml PBS.

1. Add 3ml lysis buffer to each 1 ml sample, mix by vortexing briefly (5 sec), incubate 10min at RT.
2. Add 50µl well-mixed magnetic silica, mix by vortexing briefly, incubate 10min at RT.
3. Centrifuge 2 min, 1,500 x *g,* discard supernatant carefully by e.g aspiration.
4. Add 400µl wash **buffer 1○**, resuspend the pellets by pipetting/vortexing.
5. Transfer suspensions to separate labeled 1.5ml screw-cap tubes on the miniMAG/easyMAG extraction system.
6. **Raise magnet,** allow silica to settle, wash 30sec using automated wash steps (0.5s setting). Discard supernatant by e.g aspiration.
7. **Lower magnet**, add 400µl wash **buffer 1○**.Resuspend pellet and Repeat step 6.
8. **Lower magnet**, add 500µl wash **buffer 2●**. Resuspend pellet and Repeat step 6. **Repeat**.
9. **Lower magnet**, add 500µl wash **buffer 3●**.

10. **Raise magnet,** allow silica to settle, wash 15sec using automated wash steps (0.5s setting). Discard supernatant by e.g aspiration.

11. NOTE: samples should not be left in wash buffer 3 for longer than strictly necessary.

12. **Lower magnet**, add 100µl elution buffer (**buffer 3●**). Cap tubes, transfer to thermoshaker or equivalent.

13. Incubate 5 min, 60ºC with shaking 1400 rpm.

14. Place tubes in rack, raise magnet, allow silica to settle, transfer eluates to clean tubes, retain at 4 °C for max 24 h or -80 °C for longer periods.