Appendix A

ANALYTICAL METHODS
NOROVIRUS AND ADENOVIRUS

Viruses in Raw and Partially Treated Water

Standard Operating Procedure 1:	Preparation of human norovirus standards for use as process controls
Standard Operating Procedure 2:	Preparation of adenovirus standards for use as process controls
Analytical Method 1:	Concentration of norovirus and adenovirus from raw and partially treated waters using adsorption/elution on/off a filter membrane and acidified beef extract (ABE)
Analytical Method 2:	Quantification of norovirus in water sample concentrates using reverse transcriptase polymerase chain reaction (RT-qPCR)
Analytical Method 3:	Quantification of adenovirus in water sample concentrates using quantitative polymerase chain reaction (qPCR)
Analytical Method 4:	The detection of infective adenovirus particles using Integrated Cell Culture-Polymerase Chain Reaction (ICC-PCR)

VIRUSES IN RAW AND PARTIALLY TREATED WATER

STANDARD OPERATING PROCEDURE: 1 (SOP 1)

PREPARATION OF HUMAN NOROVIRUS STANDARDS

FOR USE AS PROCESS CONTROLS

WARNING – Noroviruses are human pathogens. All samples, sample concentrates and standards (control preparations used as process controls) must be handled by trained staff in a laboratory with appropriate containment and equipment. Persons using this Standard Operating Procedure (SOP) must be familiar with routine virology laboratory practice. This SOP does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

1. AIM

To prepare a standard norovirus (NV) preparation (suspension) of intact virus particles for use as a process control during the isolation and concentration of NV from large volumes of raw and partially treated water.

2. PRINCIPLE

The preparation of a reliable/reproducible standard for use as a process control is of utmost importance when considering the isolation and enumeration of enteric viruses from water samples. Unlike many other enteric viruses, NV cannot be propagated in tissue culture and standard preparations must be extracted from human faecal material obtained from hospitals and other health institutions. These preparations may be highly variable in terms of both virus concentration and the proportion of intact virus particles present. Faecal preparations will contain a significant amount of free viral RNA and this must be removed before any estimation of the concentration of intact virus particles is made. In this way, it is possible to make an accurate assessment of the numbers of intact virus particles in the standard preparation and thus to determine the recovery rate following sample concentration and processing.

3. HEALTH & SAFETY

NV is a pathogen. Laboratory procedures should be carried out to at least ACDP Containment Level 2, with precautions taken to control significant aerosols and to avoid ingestion of contaminated material.

Only those staff who have received formal training in the application of the procedures described in this document should be permitted to undertake the analysis.

All wastes and equipment should be disinfected with Chloros/Virkon (as appropriate) before disposal or further cleaning/autoclaving.

4. EQUIPMENT

- General — all plasticware and glassware must be sterile
- Glass beads — ballotini balls, 0.5mm glass beads, 0.1mm glass beads
- Centrifuge tubes — sterile, 50 ml volume
- Top pan balance
- Electronic pipettes — Air displacement, calibrated gravimetrically with sterile disposable polypropylene filter tips
- Pipettes — sterile, volumes 1 ml to 50 ml
- Spatula — sterile
- Vortex mixer
- Incubator — capable of being maintained at 37 (±0.5)°C
- Eppendorf tubes — sterile
- Freezer — capable of being maintained at -70 (±2)°C

5. REAGENTS

- RNase solution — sterile, 100mg/ml in molecular biology grade water

6. PROCEDURE

Combine the faecal samples (provided by the Health Protection Agency (HPA) or Hospital) and transfer to a 50 ml centrifuge tube. Depending on the viscosity of the faecal samples, use either a pipette or spatula as appropriate.

6.2 Prepare a 20% (w/v) suspension of the faecal sample in sterile water:

- Add 1 ballotini ball, 0.1g of 0.5mm glass beads, 0.1g of 0.1mm glass beads.
- Vortex the suspension for 5 min and allow to stand for 1 min.
- Using an appropriately sized pipette, transfer the supernatant to a new 50 ml centrifuge tube.
- Add RNase (10µg/ml of sample) and mix by aspiration or vortexing.
- Incubate the NV preparation at 37°C for 1h.
- Dispense the NV preparation into 100µl aliquots in sterile Eppendorf tubes.
- Store at -70°C until use.

For QC purposes, once all the aliquots are frozen, remove and thaw a sample of those aliquots that were dispensed first, 25%, middle, 75% and last. Add 5.5µl EDTA to each. Extract RNA and assess concentration by qRT-PCR. Examine data for trends, each aliquot should record a comparable concentration to the others. Record data. These data are used to inform the approach to subsequent use in terms of spike concentrations and appropriate dilutions. The target number of virus particles for process controls is between 10^5 and 10^6 particles.

6.3 SPIKING WATER SAMPLES WITH NV CONTROL MATERIAL

Thaw required number of 100µl NV volumes (Eppendorf tubes)

Mix thoroughly and briefly centrifuge before addition to 5ml Ringers solution

Mix by swirling for 30 seconds and then add to 5/10 or 20 litres of relevant water matrix

Disperse spike material by mixing using an appropriate method

VIRUSES IN RAW AND PARTIALLY TREATED WATER

STANDARD OPERATING PROCEDURE: 2 (SOP 2)

PREPARATION OF ADENOVIRUS STANDARDS FOR USE AS PROCESS CONTROLS

WARNING – Adenoviruses are human pathogens. All samples, sample concentrates and standards (control preparations used as process controls) must be handled by trained staff in a laboratory with appropriate containment and equipment. Persons using this Standard Operating Procedure (SOP) must be familiar with routine virology laboratory practice. This SOP does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

1. AIM

To prepare a standard adenovirus (AdV) preparation (suspension) of intact virus particles for use as a process control during the isolation and concentration of AdV from large volumes of raw and partially treated water.

2. PRINCIPLE

AdV Type 2 NCPV#213 preparations purchased from the Health Protection Agency (HPA) are propagated on 293 cells and as such, contain intact AdV, cellular debris and free AdV DNA. To obtain a standardised AdV preparation of intact virus particles for use as a process control, the cell debris and free nucleic acid must be removed. This is achieved by filtration of the preparation (to remove cell debris) followed by DNase treatment of the filtrate (to remove free DNA).

3. HEALTH & SAFETY

AdV is a human pathogen. Laboratory procedures should be carried out to at least ACDP Containment Level 2, with precautions taken to control significant aerosols and to avoid ingestion of contaminated material.

Only those staff who have received formal training in the application of the procedures described in this document should be permitted to undertake the analysis.

All wastes and equipment should be disinfected with Chloros/Virkon (as appropriate) before disposal or further cleaning/autoclaving.

4. EQUIPMENT

- General — all plastic ware and glassware must be sterile
- Syringe filter — sterile, 0.22 µm
- Centrifuge tubes — sterile, 50 ml volume
- Electronic pipettes — Air displacement, calibrated gravimetrically with sterile disposable polypropylene filter tips

- Vortex mixer
- Incubator capable of being maintained at 37 (±0.5)°C
- Eppendorf tubes sterile
- Freezer capable of being maintained at -70 (±2)°C

5. REAGENTS

- 10x DNase buffer sterile
- EDTA 50 mM
- DNase solution sterile, 2 U/µl

6. PROCEDURE

6.1 PREPARATION OF DNase SOLUTION

6.1.1 Prepare 10X DNase buffer (100 mM Tris pH7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$).
6.1.2 Make a DNase solution of 2 U/µl DNase buffer.

6.2 PREPARATION OF AdV CONTROL MATERIAL

Adenovirus Type 2 NCPV#213 is supplied by HPA at a titre of approximately 3.0 x 10^8 pfu/ml

6.2.1 Prepare a 1:10 dilution of the AdV stock by adding 900 µl sterile PCR-grade water to 100 µl AdV stock.
6.2.2 Mix the AdV preparation by aspiration or vortexing.
6.2.3 Filter the AdV preparation using a 0.22 µm syringe filter.
6.2.4 Repeat the filter step with a new syringe filter.
6.2.5 Add DNase (Promega SV Total RNA Isolation System) to the AdV preparation at a concentration of 2 U DNase per 100 µl AdV preparation.
6.2.6 Incubate at 37°C for 10 minutes.
6.2.7 Dispense the AdV preparation into 100 µl volumes in sterile Eppendorf tubes.
6.2.8 Store at -70°C.

For QC purposes, once all the aliquots are frozen, remove and thaw a sample of those aliquots that were dispensed first, 25%, middle, 75% and last. Add 5.5µl EDTA to each. Extract DNA and assess concentration by qPCR. Examine data for trends, each aliquot should record a comparable concentration to the others, Record data. These data are used to inform the approach to subsequent use in terms of spike concentrations and appropriate dilutions. The target number of virus particles for process controls is between 10^5 and 10^6 particles.

6.3 SPIKING SAMPLES WITH AdV CONTROL MATERIAL

- Thaw required number of 100 µl AdV volumes (Eppendorf tubes) and add 11 µl EDTA (50 mM) to each.
- Vortex the suspension and briefly centrifuge. Thoroughly mix by aspiration before addition to 5ml Ringers solution
- Mix by swirling for 30 seconds and then add to 5, 10 or 20 litres of relevant water matrix
- Disperse spike material by mixing using an appropriate method

VIRUSES IN RAW AND PARTIALLY TREATED WATER

ANALYTICAL METHOD: 1

CONCENTRATION OF NOROVIRUS AND ADENOVIRUS FROM RAW AND PARTIALLY TREATED WATERS USING ADSORPTION/ELUTION ON/OFF A FILTER AND BEEF EXTRACT (ABE)

1. INTRODUCTION

More than 100 different viruses are known to be excreted in human faeces. These viruses, collectively known as enteric viruses, are generally transmitted via the faecal oral route and primarily infect and replicate in the gastrointestinal tract of the host. Enteric viruses are shed in considerable numbers (up to 10^{11} particles per gram of stool) in faeces of infected individuals, irrespective of whether or not they have clinical symptoms. Over the past 30 years, enteric viruses have been readily isolated from sewage and sewage-polluted waters throughout the world, and in environmental and potable waters that receive treated or untreated wastewater either directly or indirectly.

Noroviruses are a major cause of epidemic and endemic gastroenteritis worldwide. They are small round structured viruses, 30nm in diameter and contain a positive sense, single-stranded RNA genome. Noroviruses form the genus *Norovirus* within the Calciviridae family and genetically, are a very diverse group (Atmar and Estes, 2006). The *Norovirus* genus is divided into 5 genogroups (genogroups I-V). Human illnesses can be caused by GI, GII and GIV norovirus, while GIII and GV cause infections only in other animals. GII has been shown to account for the majority (92%) of reported human norovirus cases. Whilst most food and water outbreaks are associated with the consumption of bivalve shellfish, several waterborne outbreaks have been described. The diversity of norovirus variants increases continually due to the generation of new variants. Infections occur throughout the year, but there is a large annual peak during the cold winter months (Mounts *et al*., 2002). Infected individuals produce norovirus particles in high numbers and concentrations in stools may reach 10^{10} particles/ml. To date, human noroviruses have not been routinely cultured in the laboratory and quantification has been achieved solely using molecular biological techniques (qRT-PCR)

Adenoviruses are the second most important viral pathogens of childhood gastroenteritis (AdV 40,41) and may be excreted by healthy individuals. High concentrations are frequently detected in sewage. Human adenoviruses are 90-100nm in diameter, are members of the genus Mastadenovirus within the Adenoviridae family and contain double stranded linear DNA in a non-enveloped icosahedral shell. Infections occur throughout the world and they have been cited to cause symptom infections in several organ systems including the respiratory system (pharyngitis, acute respiratory disease and pneumonia, eye (conjunctivitis), gastrointestinal tract (gastroenteritis), the CNS (meningocepahalitis) and genitalia (urethritis and cervitis). The most common adenoviruses (adenoviruses 1,2 and 5) infect 40-60% of children, and serotypes 40 and 41 are responsible for most cases of adenovirus-associated gastroenteritis and are considered to be the second most important viral pathogen of childhood gastroenteritis after rotavirus (Crabtree *et al*., 1997). Transmission includes the faecal oral route. They are frequently detected in sewage (Jiang, 2006) and environmental waters and their high molecular weight may confer an increased resistance to environmental challenge.

This method describes the co-isolation of norovirus and adenovirus from large volumes of water and may be applied to both raw and partially treated waters.

2. PRINCIPLE

The concentration of relatively low numbers of viruses from large volumes of water generally involves two main stages:

- The reduction of the original sample volume from 5-100 litres to <30ml
- Further reduction to <10ml

Because of the small size of the virus particles, mechanical filtration is not possible, and the capture of suspended virus is effected using adsorption/elution of the virus on/off the filter matrix. This approach is based on ionic charge and involves the manipulation of changes on the surface of the virus, using pH amendment to maximize their adsorption to charged filters (Katznelson *et al*., 1976). The virus-containing sample is acidified to pH 3.2 and passed under positive pressure over a negatively charged filter (0.45µm cellulose nitrate membrane). Under acidified conditions, viruses become positively charged and are bound to the filter by electrostatic attractive forces. Bound viruses are then released from the filter by elution into a small volume of proteinaceous liquid eg beef extract or skimmed milk at high pH (9.5). Further concentration is achieved using isoelectric coagulation (flocculation) of the protein by reducing the pH to 3.5. The viruses adsorb to the floc or precipitate, which is then centrifuged to form a pellet before being dissolved in a small volume of buffer (around 4ml) for storage at -70 degrees C prior to further processing.

3. HEALTH & SAFETY

The appropriate Guidance for Safe Working Practice must be followed at all times. The concentration procedure involves the use of: heavy equipment, compressed air, centrifugation, potentially hazardous samples and a variety of reagents.

NV and AdV are both human pathogens, Microbiological hazard category 2. All samples and controls must be handled by trained staff in a laboratory with appropriate equipment. Persons using this method must be familiar with routine virology laboratory practice. This method and procedures do not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

Risk Assessment

Analysts must be familiar with the relevant risk assessments before commencing work on this method.

Specific risks identified for this method are:

1. Use of weak acid and/or alkali

2. Manual handling of;

    a. Pressure vessel (filled)

    b. Filtration tripods

    c. Sample containers containing large volumes of water

    d. Centrifuge rotor

The concentration equipment is very heavy and care should be taken when moving it

3. Pressurized vessels

4. Contact with other microbiological hazards

5. Use of autoclaves

6. Use of Chloros (CAS No. 2893-78-9)

7. Use of boiling water baths / steamer

8. Performing repetitive tasks such as pipetting and others such as standing, moving and bending

To mitigate the risks, the following recommendations are made:

1. Fill the pressure vessel with the water sample from waist height. Never lift large volumes of water from the floor.

2. Tripods may lifted from the floor only when they are positioned close to the feet.

3. Lift one water sample at a time from floor level, when between the feet.

4. The centrifuge rotor should be kept as close to the body as possible when lifting and lowering into the centrifuge. Lifting should be undertaken only when the rotor is empty, i.e. containing no canisters/centrifuge pots.

5. Ensure the correct PPE (gloves, lab coat, safety specs, safety shoes as appropriate) are worn

4. CHEMICALS/REAGENTS

Hydrochloric Acid (1M)

Buffer solutions – pH 4, 7 and 10

Sodium Hydroxide (1M)

3% Beef extract with Glycine (0.15 M)

Buffer: Disodium hydrogen orthophosphate (0.15M, pH 7.5)

5. ANALYTICAL QUALITY CONTROL

Sample storage: Once taken, samples for virological analysis should be transferred to dark storage conditions and kept at a temperature of below 15 ºC whilst awaiting processing. Samples should be concentrated as soon as practicable and preferably within 24 hours of sample collection. However, samples may be stored as described for up to 48 hours before commencing concentration.

Where possible, process control samples containing spiked viruses should be concentrated in a separate laboratory to avoid contamination. If this facility is unavailable, these samples should be processed at the end of the day when the environmental samples have been concentrated and stored.

The inclusion of process control **(positive control)** samples to demonstrate that the analytical procedure is performing within acceptable limits (in terms of recovery statistics) is extremely important in the context of this methodology. A process control sample which is of a similar matrix and volume to the water type under study should be included each time the method is undertaken. Water samples are spiked with a known number of virus particles (around 10^5-10^6), the water is then processed following the documented methodology and the recovery of the targeted viruses (%) is then calculated.

NV standardised control material (SOP 1: Preparation of human norovirus standards for use as process controls).

AdV standardised control material (SOP 2: Preparation of adenovirus standards for use as process controls).

A **negative control** or blank sample (10 litres sterile water) should be processed at the start of each sample run ie filtered using the same equipment but before the samples are processed.

6. EQUIPMENT

In addition to the general laboratory facilities and equipment, the following items of equipment are required:

Compressed Air supply (sufficient to provide 3 bar pressure (Or vacuum/peristaltic pump))	
Membrane filter holders	Stainless Steel, 142mm diameter
Pressure vessels and connecting hoses	Stainless Steel, 10 L
Cellulose Nitrate Membranes	142mm, 0.45µm porosity
Waterbath	70°C
pH Meter	
Freezer	-70°C ± 10ºC
Balance	
Cooled centrifuge	7000xg with 6 x 500ml capacity rotor
Centrifuge bottles	500 ml, sealable caps
Orbital shaker	

7. ANALYTICAL PROCEDURE

Unless otherwise specified, all liquid transfers should be made using calibrated autopipettes and mass measurements made using a top pan balance.

7.1 Preparation of work area

- Disinfect the work area with 1% chloros.
- Calibrate pH meter at room temperature using pH 7 and pH 10 buffer solutions. Check against QC pH solutions.
- Adjust Beef Extract/Glycine solution to pH 9.5±0.05 using 1M Sodium Hydroxide and 1M Hydrochloric acid immediately before use.
- Ensure pH probe is sterilised by immersing in a water bath heated to 70ºC ± 2ºC and swirling probe for 20 ± 10 seconds, before and after each use.
- Ensure Filter Housings and pressure vessels have been sterilised by autoclaving at 121ºC for 15 minutes before use.

7.2 Sample concentration

7.2.1 Acidification of sample

1. Calibrate the pH probe at room temperature using pH 7 and pH 4 buffer solutions. Check against pH QC solutions.
2. Pour the water sample (5-20 litres) into the pressure vessel
3. Process control samples: add 1-10x10^5 NV particles and/or 1-10x10^5 AdV particles prepared and stored as described in SOP 1 and 2). Mix thoroughly. Ensure that a minimum of 100 µl of the NV and AdV spikes are stored at -70°C (for subsequent RT-qPCR/qPCR quantification).
4. Sterilise the pH probe by immersing in a 70°C± 2ºC water bath, swirling the probe for 20 ± 10 seconds, before and after each use. Rinse probe in RO water before use.
5. Adjust the sample to pH 3.2 ±0.05 by the addition of 1M HCl and 1M NaOH. When the pressure vessel lid is removed, it must be held or suspended above the bench surface. Use a 50 ml disposable pipette to thoroughly mix the sample whilst adjusting the pH. Ensure that the sample is adequately mixed before each pH reading. Replace pressure vessel lid.

7.2.2 Filtration of sample (adsorption of viruses)

Assemble pressure vessel and filter housing

6. Place a 142mm, 0.45µm membrane in the filter housing. Clamp filter housing securely shut. Close all valves.
7. Turn on compressed air, adjust pressure to 1 Bar. Adjust air flow if necessary to maintain pressure at 1 Bar at all times.
8. Connect pressure hose from sample outlet valve of pressure vessel to inlet valve of filter housing. Connect air hose to air inlet valve of pressure vessel.
9. Open upper valve of filter housing to release trapped air. Close valve. Allow filtrate to go to waste.
10. When flow ceases, open lower valve to release excess water. Disconnect the pressure hose from the filter housing. Depressurise pressure vessel.
11. With the lower valve on the filter housing still open, use the lower tubing to ‘milk’ remaining liquid from filter housing.
12. If the sample volume is greater than 10 L, repeat steps 8-10 until the required total volume has been filtered.
13. A second filter may be used if necessary to process larger volumes of water where the initial filter has become blocked due to, for example, to the presence of suspended particulates.

7.2.3 Removal of viruses from filter (Elution)

14. Using a sterile measuring cylinder, pour 70±2 ml sterile 3% Beef Extract solution (pH 9.5) into the top of the filter housing.
15. Place centrifuge bottle under outlet tube and reconnect pressure hose. Collect eluted beef extract.
16. When the flow ceases, open the lower valve ensuring collection of beef extract into the centrifuge bottle. Disconnect the pressure hose from the filter housing. Depressurise pressure vessel.
17. With lower valve on filter housing still open, ‘milk’ remaining liquid from filter housing into centrifuge bottle.

7.2.4 Secondary concentration

18. Balance the sample eluants in pairs to <1 g difference using beef extract solution.
19. Calibrate the pH probe at room temperature using pH 7 and pH 4 buffer solutions. Check against pH QC solutions.
20. Adjust eluent to pH 3.3 ±0.05 using 1 M HCl and 1M NaOH.
21. Place on orbital shaker for 5±1 min until the formation of a dense precipitate or floc. .
22. Balance samples with sterilised Millipore water to <0.1 g difference.
23. Centrifuge at 7000 x g for 20 min at 4±1ºC. Remove samples from the centrifuge immediately at the end of the cycle.
24. Carefully pour off the supernatant and redissolve the precipitate in 4±0.5 ml of 0.15 M disodium hydrogen orthophosphate by returning the centrifuge bottle to the orbital shaker until the precipitate has completely dispersed.
25. Pipette 2x100 µl virus concentrate into 2x2ml PCR-grade microtubes (one for RNA extraction for subsequent qRT-PCR NV assay, one for direct qPCR AdV assay). Pipette the remaining virus concentrate into 7 ml bijou bottles.
26. Store at -70°C $\pm$ 10°C until further analysis.

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SOP1: Preparation of human norovirus standards for use as process control material

SOP2: Preparation of adenovirus standards for use as process control material

VIRUSES IN RAW AND PARTIALLY TREATED WATER

ANALYTICAL METHOD 2

QUANTIFICATION OF NOROVIRUS IN WATER SAMPLE CONCENTRATES USING REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-qPCR)

1. INTRODUCTION

Noroviruses (NV) are the most common cause of acute gastroenteritis in humans. These viruses can be subdivided into five genogroups: GI, GII, GIII, GIV and GV. Genotypes I, II, IV are associated with infections in humans with GII being the predominant cause of NV outbreaks; GII is the target genogroup for the method described here although the principle can be applied to any genogroup.. The detection and quantification of NV in water sample concentrates using quantitative reverse transcription Polymerase Chain Reaction (RT-qPCR) is highly specific and sensitive. Using GII-specific primers and probe, it is possible to amplify NV RNA fragments directly from extracted RNA. The RT-qPCR assay uses primers/probe as described by Loisy *et al* (2005). Samples for qRT-PCR analysis are prepared by RNA extraction from the sample concentrates using TRIzol Plus RNA Purification Kit (Life Technologies).

Interference

Sample concentrates may contain substances that are co-extracted with RNA and can inhibit the subsequent RT-qPCR assay. The presence of inhibitors can affect the ability to determine actual NV copy number in the sample. Thus, tenfold dilutions of the test samples are used to attempt to minimise the effect of inhibition.

2. PRINCIPLE

In the One step TaqMan® RT-qPCR system (Life Technologies), NV RNA is reverse transcribed to complementary DNA (cDNA) and a genogroup-specific DNA fragment is amplified using sequence-specific oligonucleotides and a fluorescently labelled target-specific probe. Quantification of NV cDNA in the test sample is achieved by measuring the fluorescence level during each PCR cycle. An increase in the product during qPCR causes a proportional increase in fluorescence due to the breakdown of the probe and the release of the fluorophore. For every positive reaction, a Ct (threshold cycle) value is determined and this represents the cycle number of the PCR at which the fluorescence rises above a threshold level. This level can be automatically set by the qRT-PCR software but should fall within the exponential region of the amplification curves. A serial dilution of a known concentration of the target DNA fragment is used to produce the standard curve. The copy number of NV in the sample is then determined by comparison of the Ct values obtained for sample concentrates to those of the standard curve.

The preparation of sample concentrates for RT-qPCR is of critical importance. NV concentrations in raw and partially treated water samples may be very low, and sample concentrates may contain substances that are inhibitory to RT-qPCR. Thus, in order to produce a pure RNA sample for subsequent analysis, it is prudent to incorporate an RNA extraction step. TRIzol Plus RNA Purification Kit (Life Technologies) uses TRIzol reagent, a monophasic solution of phenol and guanidine isothiocynate which disrupts the cells and dissolves cell components whilst maintaining RNA integrity. The addition of chloroform and centrifugation separates the solution into an upper aqueous phase containing RNA and a lower phenol-containing organic phase. The

organic phase is discarded and the upper phase is further processed and purified using a silica-cartridge purification protocol.

3. HEALTH & SAFETY

A high standard of hygienic practice must be exercised in accordance with the laboratory's Safety Code of Practice. Due to the sensitivity of the qRT-PCR assays, extra attention must be paid to avoid sample contamination during processing. Nitrile gloves must be worn at all times.

Noroviruses are human pathogens and all samples and control preparations should be handled accordingly.

After the lysis step, any possible infective material in the sample should be biologically inactive.

Only staff who have received formal training in the application of the procedures described in this document should be permitted to undertake the analysis. For specific hazards associated with the reagents used in this method, see the COSHH assessment form.

Risk Assessment

Analysts must be familiar with the relevant risk assessments associated with this method before commencing work.

Specific risks identified as relevant to this method are:

1. Use of TRIzol reagent (TRIzol Plus RNA Purification Kit, Life Technologies) which contains phenol and guanidine isothiocynate (GITC) – see COSHH form.

2. Use of chloroform – see COSHH form.

3. Use of Lysis Buffer and Wash Buffer I (TRIzol Plus RNA Purification Kit, Life Technologies) which contain guanidine isothiocynate – see COSHH form.

4. Solutions containing ethanol are considered flammable. Use appropriate precautions when using this chemical.

5. Dispose of the buffers and chemicals in appropriate waste containers.

4. CHEMICALS/REAGENTS

Ethanol	96-100% and 70% (in RNase-free water)
Chloroform	
Molecular grade water	
TRIzol Plus RNA Purification Kit	Life Technologies
TaqMan One Step RT-qPCR Mastermix	Life Technologies
1 X TE Buffer	

STANDARD SOLUTIONS

Quantified control/standard NV GII oligonucleotide DNA solutions (plasmid or oligonucleotide) according to the consensus sequence of e.g. ENA accession numbers AB447432, AB447436, AB447446, FJ537135, FJ537136, FJ514242)

Forward and reverse NV GII primers	(e.g. Loisy *et al.,* 2005)
TaqMan® reporter probe for NV GII	(e.g. Loisy *et al.,* 2005)

5. ANALYTICAL QUALITY CONTROL

All RT-qPCR plates must include standard control oligonucleotides (10^1 to 10^4).

All equipment and apparatus must be monitored to ensure correct operation.

All samples are tested in triplicate wells.

All samples are diluted to 10^{-1} for inhibition control.

All RT-qPCR plates must include No Template Control (NTC) wells for the control of contamination of qRT-PCR reagents and set up conditions.

Virus concentrates can be stored at -70 °C ±10 °C for up to 12 months. Extracted RNA from samples can be stored at -70 °C ±10 °C for up to 12 months.

6. APPARATUS

In addition to standard laboratory facilities and equipment, the following are required:

Electronic pipette	Air displacement (calibrated gravimentrically) with sterile disposable polypropylene filter tips.
Vortex mixer	
Timer	
Microcentrifuge	Capable of centrifuging at 12,000 x g
Microcentrifuge tubes	1.5 mL and 2 mL RNase-free and Dnase-free
Ultra violet light sterilisation cabinet	
Bijoux	7 mL, sterile
96-well PCR plates	
Optically clear sealing film	

7. ANALYTICAL PROCEDURE

7.1 RNA EXTRACTION

Preparation of Wash Buffer II with Ethanol

Before commencing lysis, add 60 mL 96-100% ethanol to Wash Buffer II. Store Wash Buffer II with ethanol at room temperature.

Lysate Preparation with TRIzol Reagent

Add 1 mL TRIzol reagent to 100 µL sample concentrate. Vortex and incubate at room temperature for 5 min to allow complete dissociation of protein complexes.

Phase Separation

1. Add 0.2 mL chloroform to lysate/TRIzol reagent. Shake the tube vigorously by hand for 15 seconds.

2. Incubate at room temperature for 2-3 min.

3. Centrifuge at 12,000 x g for 15 min.
(Note: After centrifugation, the mixture separates into a lower, red phenol-chloroform phase, an interphase and a colourless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is ~600 µL).

4. Transfer ~600 µL of the colourless, upper phase to a new RNase-free tube, taking care to avoid the interphase.

5. Add an equal volume of 70% ethanol to obtain a final ethanol concentration of 35%. Mix well by vortexing.

6. Invert the tube to disperse any visible precipitate that may form following addition of ethanol.

7.2 Binding, washing and elution

1. Transfer up to 700 µL of sample to a spin cartridge with a collection tube.

2. Centrifuge at 12,000 x g for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.

3. Repeat steps 1-2 until the entire sample has been processed.

4. Add 700 µL Wash Buffer I to the spin cartridge. Centrifuge at 12,000 x g for 15 s at room temperature. Discard the flow-through and the collection tube. Insert the spin cartridge into a new collection tube.

5. Add 500 µL Wash Buffer II with ethanol to the spin cartridge. Centrifuge at 12,000 x g for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into a new collection tube.

6. Repeat step 5 once.

7. Centrifuge the spin cartridge and collection tube at 12,000 x g for 1 min at room temperature to dry the membrane with attached RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.

8. Add 100 µL RNase-free water to the centre of the spin cartridge.

9. Incubate at room temperature for 1 min.

10. Centrifuge the spin cartridge with the recovery tube for 2 min at ≥12,000 x g at room temperature. Discard the spin cartridge. The recovery tube contains the purified total RNA.

11. Store RNA preparation at -70 °C.

7.3 RT-qPCR

RT-qPCR set up

- Prepare 10-fold dilution of the RNA preparations by adding 10 µL RNA preparation to 90 µL sterile molecular grade water.

- Sterilise a complete set of equipment (electronic pipettes and tips: 10, 120, 300, 1000 µL; 96-well PCR tray; reagent reservoir, disposable 7 ml bijou bottle; molecular grade water) by exposure to UV light for 20 min in the UV light sterilisation cabinet.

- Thaw forward and reverse primers, probe and standard controls before use.

- Refer to mastermix calculator (appendix 2) for preparation of correct volume of mastermix, depending on how many wells of the 96-well PCR tray will be used.

- Combine all reagents (water, master mix, Rnase inhibitor, primers, probe) in a 7 mL sterile disposable bijou bottle. Mix by aspirating 3 times with an electronic 1000 µL pipette.

- Dispense 20 µL of mastermix into each well required of the 96-well tray.

- Add 5 µL molecular grade water to the NTC wells.

- Add 5 µL sample RNA preparation to appropriate wells (in triplicate).

- Add 5 µL of each control standard to appropriate wells (in duplicate).

- Seal the plate with sealing film and smooth down evenly.

- Spin the plate for 2 min at 850 x g.

- Heat seal the plate for 15 s using the electronically heated plate sealer.

Operation of qPCR machine (dependent on qPCR machine used)

1. Load the plate into the qPCR machine.

2. Select Unknown, NTC and Standard well types according to template sheet. For each well type, also select reporter and reference fluorescent dyes (with ROX usually being the reference dye).

3. Label sample wells according to sample being tested.

4. Cycling conditions are as follows: 48 °C 15 min; 95 °C 10 min; 95 °C 15s, 60 °C 1 min for 45 cycles.

5. On completion of qRT-PCR assay, check that the standard curve meets the following criteria: slope -3.10 to -3.92, R-sq >0.9 and efficiency 80-110%. Check NTCs have no Ct value.

Provided the NTC and laboratory blank controls are satisfactory, and the standard curve fits within the acceptable limits, use the values obtained per well to calculate the number of norovirus genome copies in the original water sample. The calculation should account for the volume of RNA template (and dilution factor, if any); the volume of sample concentrate and the initial volume of water processed to obtain the concentrate.

REFERENCES

Loisy, F, R. L. Atmar, P. Guillon, P. Le Cann, M. Pommepuy and F. S. Le Guyader. 2005. Real-time RT-PCR for norovirus screening in shellfish. Journal of Virological Methods 123: 1-7.

VIRUSES IN RAW AND PARTIALLY TREATED WATER

ANALYTICAL METHOD 3

QUANTIFICATION OF ADENOVIRUS IN WATER SAMPLE CONCENTRATES USING QUANTITATIVE POLYMERASE CHAIN REACTION (q-PCR)

1. INTRODUCTION

Adenoviruses (AdV) are the second most important viral pathogens of childhood gastroenteritis (AdV40,41) and may be excreted by healthy individuals. They are extremely abundant in sewage and are frequently detected in environmental waters independently of season and location. AdV are environmentally resistant and have been shown to display greater resistance to UV treatments because they contain double-stranded DNA, which allows for repair of damaged DNA using the enzymes of host cells. In addition, their high molecular weight may confer an increased resistance to environmental conditions.

AdV detection in virus concentrates from water samples using quantitative Polymerase Chain Reaction (qPCR) is a highly specific and sensitive detection method. Using AdV-specific primers and probes it is possible to amplify AdV DNA fragments directly from virus concentrates. This qPCR assay uses primer/probe as described by Hernroth *et al.,* (2002).

2. PRINCIPLE

An AdV-specific DNA fragment is amplified using sequence-specific primers and a fluorescently labelled, target-specific probe. Quantification of AdV DNA in a test sample is achieved by measuring the fluorescence level during every PCR cycle. An increase in the product during qPCR causes a proportional increase in fluorescence due to the breakdown of the probe and the release of the fluorophore. An amplification-based critical threshold (Ct) is calculated using an algorithm which determines the portion of the amplification plots where all of the data curves display an exponential increase in fluorescence. A serial dilution of a known concentration of the target DNA fragment is used to produce a standard curve. The copy number of AdV in the sample is determined and quantified by comparison of Ct values obtained for sample concentrates with the standard curve.

AdV quantification is obtained by direct qPCR of the virus concentrate (without extraction) and it is possible that substances inhibitory to the PCR process are concentrated along with the viruses. The presence of these inhibitors can affect the ability to determine actual AdV copy number in the sample. Tenfold dilution of the test samples (sample concentrates) is used to dilute the effect and to detect any inhibition of this nature.

3. HEALTH & SAFETY

A high standard of hygienic practice must be exercised in accordance with the laboratory's Safety Code of Practice. Due to the sensitivity of the qPCR assays, extra attention must be paid to avoid contamination of sample concentrates during processing. Nitrile gloves must be worn at all times.

Adenoviruses are human pathogens and all samples and control preparations should be handled accordingly.

Analysts must be familiar with the relevant risk assessments before commencing work on this method.

4. REAGENTS

Molecular grade water

TaqMan PCR Mastermix reagent Life Technologies

1 X TE Buffer

Bovine serum albumin (4% filter sterilised solution)

5. STANDARD SOLUTIONS

Quantified control/standard AdV DNA suspensions (plasmid or oligonucleotide) according to the DNA sequence of e.g. ENA accession number J01917

Forward and reverse AdV primers (e.g. Hernroth *et al.,* 2002)

TaqMan reporter probes for AdV (e.g. Hernroth *et al.,* 2002)

6. ANALYTICAL QUALITY CONTROL

- All qPCR plates must include standard control oligonucleotides (10^1 to 10^4).
- All equipment and apparatus must be monitored to ensure correct operation.
- All samples are tested in triplicate wells.
- All samples are diluted to 10^{-1} for inhibition control.
- All qPCR plates must include No Template Control (NTC) wells for the control of contamination of qPCR reagents and set up conditions.
- Virus concentrates can be stored at -70 °C ±10 °C for up to 12 months.

7. APPARATUS

In addition to standard laboratory facilities and equipment, the following are required:

- Electronic pipette — Air displacement (calibrated gravimentrically) with sterile disposable polypropylene filter tips.
- Vortex mixer
- Timer
- Microcentrifuge — Capable of centrifuging at 12,000 x g
- Microcentrifuge tubes — 1.5 mL, DNase-free
- Ultra violet light sterilisation cabinet
- Bijoux — 7 mL, Sterile
- 96-well PCR plates
- Optically clear sealing film

8. ANALYTICAL PROCEDURE

8.1 q-PCR

q-PCR set up

1 Prepare 10-fold dilution of the virus concentrates by adding 10 µL concentrate to 90 µL sterile molecular grade water.

2 Sterilise a complete set of equipment (electronic pipettes and tips: 10, 120, 300, 1000 µL; 96-well PCR tray; reagent reservoir, disposable 7 ml bijou bottle; molecular grade water) by exposure to UV light for 20 min in a UV light sterilisation cabinet.

3 Thaw forward and reverse primers, probe and standard controls before use.

4 Refer to mastermix calculator (Appendix 2) for preparation of correct volume of mastermix, depending on how many wells of the 96-well PCR tray will be used.

5 Combine all reagents (water, master mix, BSA, primers and probe) in a 7 mL sterile disposable bijou bottle. Mix by aspirating 3 times with an electronic 1000 µL pipette.

6 Dispense 23 µL of mastermix into each well of the 96-well tray.

7 Add 2 µL molecular grade water to the NTC wells.

8 Add 2 µL virus concentrate to appropriate wells (in triplicate).

9 Add 2 µL of each control standard to appropriate wells (in duplicate).

10 Seal the plate with sealing film and smooth down evenly.

11 Spin the plate for 2 min at 850 x g.

12 Heat seal the plate for 15 s using the electronically heated plate sealer.

Operation of qPCR machine (variable depending on equipment used)

1 Load the plate into the qPCR machine.

2 Select Unknown, NTC and Standard well types according to template sheet. For each well type, also select reporter and reference fluorescent dyes (with ROX usually being the reference dye).

3 Label sample wells according to sample being tested.

4 Cycling conditions are as follows: 50 °C 10 min; 95 °C 10 min; 95 °C 15s, 59 °C 1 min for 45 cycles.

5 On completion of qPCR assay, check that the standard curve meets the following criteria: slope - 3.10 -3.92, R-sq >0.9 and efficiency 80-110%. Check NTCs have no Ct value.

Provided the NTC and laboratory blank controls are satisfactory, and the standard curve fits within the acceptable limits, use the values obtained per well to calculate the number of adenovirus genome copies in the original water sample. The calculation should account for the volume of DNA template (and dilution factor, if any); the volume of sample concentrate and the initial volume of water processed to obtain the concentrate.

REFERENCES

Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK. 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68:4523-33.

VIRUSES IN RAW AND PARTIALLY TREATED WATER

ANALYTICAL METHOD 4

THE DETECTION OF INFECTIVE ADENOVIRUS PARTICLES USING INTEGRATED CELL CULTURE-POLYMERASE CHAIN REACTION-PCR (ICC-PCR)

1. INTRODUCTION

Currently, the combination of PCR with laboratory cell culture offers the best approach to assess viral infectivity, including the detection of slow growing fastidious viruses such as human adenoviruses. This technique, Integrated Cell Culture-PCR (ICC-PCR) permits the detection of infectious virus where the virus normally fails to produce visual cytopathic effect (CPE) in mammalian cell culture or where CPE takes a long time to appear. The technique relies upon an initial biological amplification of viral nucleic acid in cell culture, followed by amplification via PCR. This method is rapid and sensitive, semi-quantitative and potentially suitable for routine use for the detection of infectious adenovirus (AdV) in concentrates of processed water samples. Sample concentrates may be screened by qPCR before application of this method to establish the likely presence of target virus.

2. PRINCIPLE

A monolayer of A549 cells is grown until the cell sheet is confluent. The growth medium is removed and the test material (sample concentrate) is added to the cells. The flask is incubated for 1 hour to allow any viruses present in the sample concentrate to attach to the cells and then the inoculum is discarded. The cells are washed to remove any remnants of the inoculum. Fresh maintenance medium containing guanidine hydrochloride (GuHCL), which inhibits the replication of enteroviruses but does not affect the replication of AdV, is added to the flask. The flasks are then incubated for five days before analysis of the flask contents for the presence of infective viruses. DNA is purified from a total cell and viral particle lysate using DNeasy Blood and Tissue Kit (Qiagen) and used for qPCR. AdV-specific primers and probes (Hernroth *et al,* 2002) are used to amplify the AdV fragments. Appropriate controls are included to check for residual inoculum.

ICC-PCR may be used to assess the virus content of an inoculum/sample either in terms of presence/absence or as a semi-quantitative measure of the number of infective viruses present. A semi-quantitative estimate of virus numbers may be achieved by inoculating flasks containing confluent A549 cell monolayers with a range of AdV concentrations (eg 10- 10^5 $\log_{10}$ HPA stock; T_0: initial inoculum)). The flasks are incubated for 5 days (T_5). The levels of virus (as measured by qPCR) are recorded at T_0 and T_5 and the increase in virus levels (T_5-T_0) following incubation for each inoculum level is compared to the initial inoculum level. Linear regression may then be used to estimate the original level of viruses in a sample concentrate. An example of this is achieved is presented in Appendix 2.

3. HEALTH & SAFETY

Adenoviruses are human pathogens. All samples and control preparations must be handled by trained staff in a laboratory with appropriate equipment. Persons using this Method must be familiar with routine virology laboratory practice and a high standard of hygienic practise must be observed at all times. This method document does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

3.1 Microbiological hazard classification: Category 2

3.2 Risk Assessment

Analysts must be familiar with the relevant risk assessments before commencing work.

Specific risks associated with this method are:

1. Contact with microbiological hazards

2. Use of autoclaves

3. Use of Chloros (CAS No. 2893-78-9; sodium hypochlorite, 1%)

4. Use of guanidine hydrochloride

5. Manual handling of centrifuge rotor

To mitigate the risks, the following recommendations are made:

1. Ensure correct PPE (gloves, lab coat, safety specs, safety shoes)
2. Use ethanol to clean up small spills
3. Use Chloros for contaminated waste that contains guanidine hydrochloride.

Autoclave contaminated glassware/tissue/gloves etc

4. CHEMICALS

Ethanol	96-100%
Sodium hypochlorite	1%

5. REAGENTS/MEDIA

A549 Growth Medium	Appendix 1: A1
A549 Maintenance Medium	Appendix 1: A2
DNeasy Blood and Tissue Kit	Section 8.3
Phosphate-buffered saline (PBS)	Appendix 1: A3
Trypsin solution	Appendix 1: A4

Tissue culture media and all other reagents must be suitable for the culture and maintenance

of A549 cells. Growth and Maintenance media (MEM) may be purchased ready to use or made in-house using a defined formulation (Appendix 1).Foetal Calf Serum along with the other components are added to the MEM ready for use.

6. **STANDARDS:** AdV standardised control material (SOP:2: Preparation of adenovirus standards for use as process controls)

7. **EQUIPMENT**

In addition to the standard laboratory facilities and equipment, the following are required:

• Incubator	capable of being maintained at (37±2)°C
• Refrigerator	capable of being maintained at (4±3)°C
• Freezer	capable of being maintained at (-70±5)°C
• Tissue culture flasks	sterile, 25 cm^2 (assay) 150cm^2 (production)
• Pipettes	volumes 1 ml and 10 ml, sterile, graduated
• Automatic pipettor	
• Micropipette	volumes 1ml to 5 ml, sterile pipette tips
• Vertical laminar air-flow cabinet	for cell culture procedures
• Centrifuge	with rotor for 15 mL centrifuge tubes, capable of centrifuging at 175 x g
• Vortex mixer	
• Microcentrifuge tubes	1.5 mL and 2 mL, DNase-free
• Microcentrifuge	capable of centrifuging at 10,000 x g to 20,000 x g
• Thermomixer	heating at 37 °C

All plastics and glassware must be sterile

8. ANALYTICAL PROCEDURE

8.1 CELL CULTURE

Tissue culture is a demanding and labour intensive technique generally undertaken in specialist laboratories. However, with the appropriate time, expertise and laboratory facilities, cells may be propagated successfully in house and stored long term in liquid nitrogen. In this way, A549 cells for use in the ICC-PCR assay are available as and when required. Where laboratory facilities and/or expertise are limited, cells may be obtained as 'assay ready' (supplied as confluent or near confluent monolayers in 25cm^2 flasks by arrangement) from the HPA Culture Collections, Porton Down.

8.1.1 In house preparation of cell monolayers

Stock culture

Grow stock A549 cells on a seven-day cycle using laboratory cell culture protocols. Growth medium (Appendix 1 (A.1) is replaced with maintenance medium (Appendix 1: A.2) on day three or four.

Preparation of cells for virus assay

Prepare a fresh cell suspension from stock A549 monolayers by conventional trypsination and re-suspend the cells to give a final concentration of 1 x 10^5 per ml in Growth Medium to produce a confluent cell sheet in 3-4 days or 3 x 10^5 per ml in Growth Medium to produce a confluent cell sheet in 24 hours. Seven flasks (4 flasks per sample concentrate, 3 controls) are needed for each sample concentrate tested. Where more than 1 sample concentrate is analysed per batch, only 1 set of control flasks is required.

1. Add 5ml of this cell suspension to each 25cm^2 flask for the virus assay (making a total of 5 x 10^5 cells or 1.5 x 10^6 in each flask).

2. Incubate for 3-4 days or overnight when the cell sheet should be 90-100% confluent and can be used in the Virus Infectivity Assay.

Obtaining 'assay ready' cells

'Assay ready' A549 cells grown to confluency, can be ordered from the HPA, Porton Down. At least one week's notice is required between order and receipt of cells.

8.2 VIRUS INFECTIVITY ASSAY

8.2.1 Preparation

1. Thaw an appropriate volume of AdV standardised control material. Prepare an AdV positive control inoculum of an appropriate concentration in PBS.

2. Thaw sample concentrate known to be adenovirus positive by PCR (4ml)

3. Label 4 flasks of confluent A549 cells per sample:
   - 1 flask: (sample number) T_0

   - 3 flasks: (sample number) T_5

4. Per infectivity assay run, label 3 flasks of confluent A549 cells:
   - 1 flask: positive QC T_0

   - 1 flask: positive QC T_5

   - 1 flask: negative QC T_5

8.2.2 Inoculation Procedure

1. Pour away growth medium from each flask.

2. To the cell culture flasks add:

   - 1ml of sample concentrate to each of 4 flasks

   - 1ml AdV standardised control material to each of 2 positive QC flasks

   - 1ml PBS to 1 negative QC flask

* In order: Negative control sample, followed by samples, followed by positive control samples

3. Lay flasks flat so that the inoculum covers the cell sheet and incubate for 1hr at 37 °C.

4. Pour off inocula as above, wash cells twice with 4ml warm (37 ºC) PBS. Discard PBS carefully. Take care not to cross-contaminate flasks with medium.

5. Add 4ml of cell culture maintenance medium to each of the flasks.

6. Incubate three flasks of sample, one positive control and one negative control flask (labelled T_5) at 37 °C for five days. Freeze after five days incubation.

7. Freeze the sample control and positive QC control flasks immediately (labelled T_0).

8.2.3 Analysis

1. Freeze and thaw all tissue culture flasks three times

2. Transfer the contents (cell debris and supernatant) of each flask to a centrifuge tube and centrifuge at 175 x g for 5 minutes

3. Store 2 x 2ml volumes of each supernatant at -20 ^{o}C for further assays if required

4. Use a known volume of each supernatant for DNA extraction (8.3) and analyse undiluted by qPCR

5. Score the results positive or negative for each flask and for each dilution

The T_0 flasks must be assayed as above to determine the level of residual virus inocula attached to the cells. If either the sample or the positive QC virus is positive at T_0, the nucleic acid should be diluted and re-assayed

8.3 DNA EXTRACTION USING DNeasy BLOOD AND TISSUE KIT (QIAGEN)

Prior to starting DNA extraction

1. Add the appropriate volume of ethanol (96-100%) to Buffers AW1 and AW2 as indicated on the bottle to obtain a working solution.
2. Buffer AL may form a precipitate upon storage. If necessary, warm to 56 ºC until the precipitate has fully dissolved.

DNA extraction

1. Freeze - thaw cell culture flasks three times.
2. Transfer cell debris and supernatant to a centrifuge tube and centrifuge at 175 x g for 5min.
3. Remove 500 µL of each supernatant for DNA extraction (supernatant may be stored at -70 ºC). Store remaining supernatant at -70 ºC.
4. Add 50 µL proteinase K to 500 µL supernatant. Mix thoroughly by vortexing. Incubate using a thermomixer at 37 ºC for 10 min at 350 rpm.
5. Add 500 µL Buffer AL and mix thoroughly by vortexing.

6. Add 500 µL ethanol (96-100%) and mix thoroughly by vortexing.
7. Pipet 650 µL of the mixture from step 6 into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at 10,000 x g for 1 min. Discard flow-through.
8. Repeat step 7 until all the mixture has been passed through the spin column. Discard all flow-through and discard collection tube on final spin.
9. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1. Centrifuge at 10,000 x g for 1 min. Discard flow-through and collection tube.
10. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge at 20,000 x g for 3 min to dry the DNeasy membrane. Discard the flow-through and collection tube.

(It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent qPCR. Following the centrifugation step, remove the spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube then reuse it in another centrifugation at 20,000 x g for 1 min.)

11. Place the spin column in a sterile DNase-free 1.5 mL microtube and pipet 50 µL Buffer AE directly onto the membrane.
12. Incubate at room temperature for 1 min.
13. Centrifuge at 10,000 x g for 1 min to elute.
14. Store DNA preparation at -70 ºC until analysed using the AdV qPCR assay (Method: Quantification of adenovirus in water sample concentrates using qualtitative polymerase chain reaction). Use DNA preparation as virus concentrates.

REFERENCES

Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK. 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68:4523-33.

Appendix 1

Media : Suggested components for cell culture media

Commercial 'ready-to-use' media may be used

A.1 A549 Growth Medium	**Total Volume**		
	100 ml	500 ml	Final concentration
Sterile Deionised Water	80	400	
MEM (10x)	10	50	1x
Foetal Calf Serum (FCS)	10	50	10 %
L-glutamine (29 g/l)	1	5	2.9 mg/ml
Sodium bicarbonate ($NaHCO_3$) (4,4%)	2,5	12,5	0.11%
Penicillin & Streptomycin			
(10 000 units/ml & 10mg/ml)	1	5	100µ/ml, 0.1mg/ml

Store at (5 ± 3)°C for up to one week.

A.2 A549 Maintenance Medium	**Total Volume**		
	100 ml	500 ml	Final concentration
Sterile De-ionised Water	80	400	
MEM (10x)	10	50	1x
Foetal Calf Serum (FCS)	2	10	2 %
L-glutamine (29 g/l)	1	5	2.9 mg/ml
Sodium bicarbonate (NaHCO3) (4,4%)	4	20	0.11%
Penicillin & Streptomycin			
(10 000 units/ml & 10 mg/ml)	1	5	100units/ml, 0.1mg/ml
Nystatin	1	5	100units /ml
Guanidine hydrochloride	1	5	100ug/ml

(MP Biomedical: Catalogue number 820512)

Additional antibiotics such as gentamicin (40mg/ml final concentration) may be used if

the sample concentrate is thought to be highly contaminated. Store at (5 ± 3)°C for up to one week.

Stock Guanidine hydrochloride does not need to be sterilized but should be stored in the dark at room temperature.

A.3 Phosphate Buffered Saline (PBS)

10 Dulbecco PBS tablets

1 litre deionised water

Or

2 Invitrogen or Gibco PBS tablets

1 litre deionised water

Dispense into 500ml aliquots and autoclave at 121^0C for 15 min

A.4 Trypsin solution

Trypsin is supplied at a concentration of 25g/litre and stored at -20^0C.

Working stock and in-use stock remains at 25g/litre, in-media (as used) the concentration is 1.25mg/ml. Trypsin 'in-use' should be stored at 4^0 C.

APPENDIX 2: EXAMPLE ESTIMATION OF INFECTIVE VIRUS PARTICLES

Table A2.1. Example raw data, AdV quantification by qPCR

	AdV particles (qPCR)					
Inoculum (T_0)	Incubation time (days)				Mean (SD)	T_5-T_0
	0	5	5	5		
1.2e02	3.6e02	2.7e04	1.1e05	2.8e05	1.4e05 (1.3e05)	1.36e05
5.5e02	5.0e02	1.1e08	6.3e07	2.0e07	6.6e07 (4.7e07)	6.55e07
7.0e03	4.9e03	2.0e08	5.7e08	2.2e08	3.3e08 (2.1e08)	3.3e08
9.0e04	1.8e03	4.6e09	4.3e09	3.5e09	4.1e09 (6.0e08)	4.1e09
1.3e05	2.3e04	1.9e10	2.1e10	not done	2.0e10 (1.2e09)	1.98e10

Table A2.2. Example data, $\log_{10}$ transformed, AdV quantification by qPCR

	Log_{10} AdV particles					
Inoculum (T_0)	Incubation time (days)				Mean	T_5-T_0
	0	5	5	5		
2.09	2.55	4.43	5.02	5.44	5.135	5.134
2.74	2.70	8.06	7.80	7.29	7.816	7.816
3.85	3.69	8.31	8.76	8.35	8.522	8.522
4.95	3.26	9.67	9.63	9.54	9.616	9.616
5.10	4.36	10.28	10.32	not done	10.298	10.298

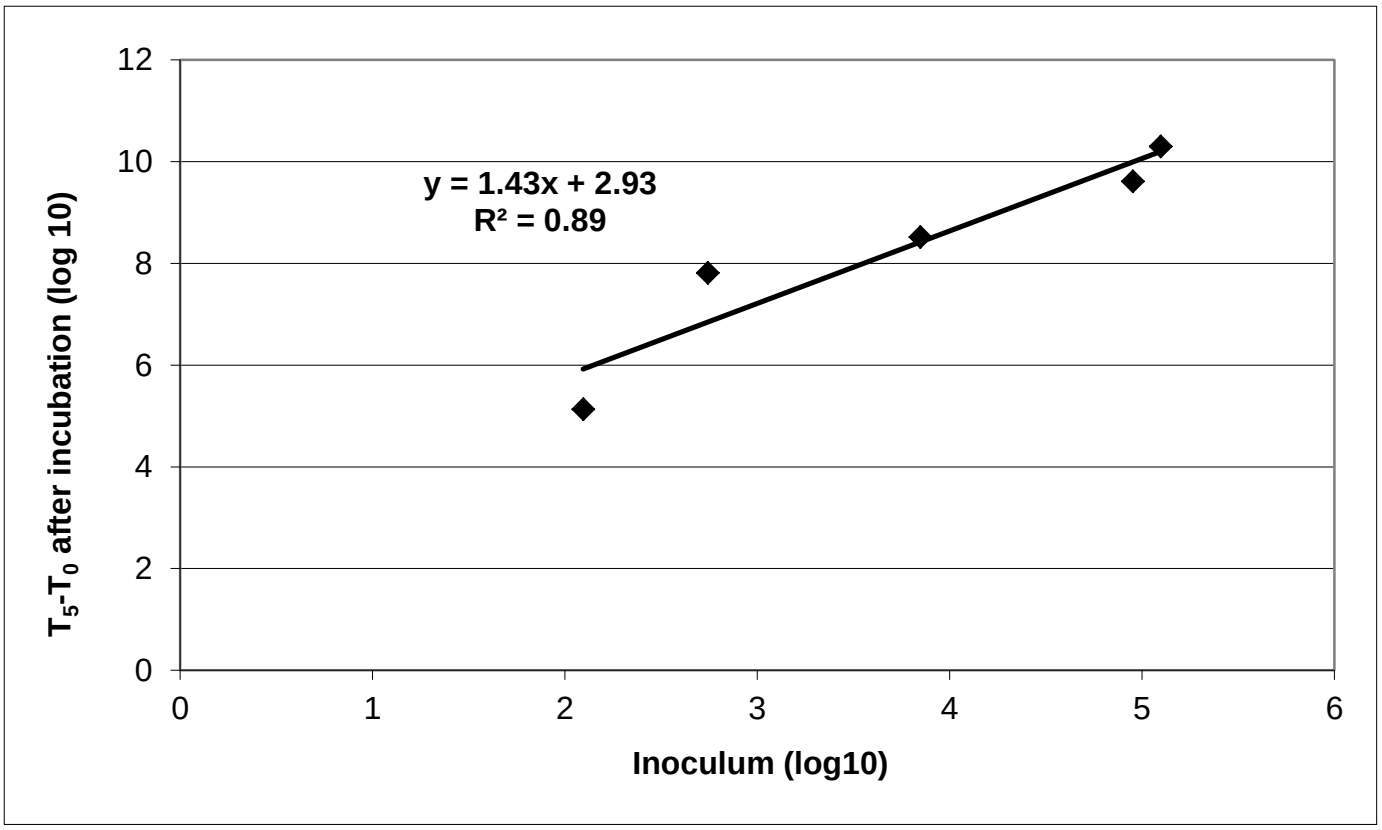

Figure A2.1. Example data plot with linear regression line, showing the relationship between increase in AdV genome number after infection with initial inoculum density. Data plotted are the initial inoculum density estimated by qPCR (column 1, Table A2.2) vs. increase in AdV genome numbers after 5 days incubation (T_5 – T_0; final column, Table A2.2).

Appendix B

METHOD SUMMARIES

- MICROBIOLOGICAL PARAMETERS
- CHEMICAL PARAMETERS

Microbiological Parameters

Method Summary for the Determination of pH, Electrical Conductivity and Turbidity in Waters by Automated Analysis

Method Summary for the Determination of Suspended Solids by Filtration

Method Summary for the Determination of Colour in Waters by Spectrophotometer

Method Title	Analyte	Accreditation	Matrix	Summary
NLS B Clost Pot	*Clostridium perfringens* Sulphite Reducing Clostridia	UKAS	Potable Water	The tests for sulphite-reducing clostridia play only a subsidiary role in water examination. These organisms form spores which are resistant to heating compared with vegetative cells and advantage is taken of this for the detection of clostridia in water. *Cl. perfringens*, an important member of this group, is associated with faecal contamination; if it is found at a time when other faecal indicator organisms are no longer detectable, it indicates remote or intermittent pollution. Samples should be analysed within 24 hours of sampling.
				Preliminary heat treatment is used to destroy vegetative bacteria if required. Measured volumes of sample are filtered through a 0.45-µm pore size membrane filter which is then transferred to a culture medium. Anaerobic incubation encourages the growth of Clostridia bacteria as black colonies on the filter. Presumptive sulphite reducing clostridia colonies are recognised by their black colour and are 2-4mm in diameter. Presumptive colonies may be subjected to further tests to confirm their identity if required. From knowledge of the volume of sample filtered and the number of colonies counted the concentration of sulphite reducing Clostridia in the original sample can be calculated. Limit of detection is 1 organism in the volume analysed. (<1 cfu/100ml) Typical reporting volume cfu/100ml. Results are available 2-3 days after analysis. For Sediments / soil a known mass of sediment / soil is measured and a known quantity of diluent is added. This is then mixed and the resulting suspension analysed.

Method Title	Analyte	Accreditation	Matrix	Summary
NLS B Coli Pot	Total Coliforms T Coli Coliforms	UKAS	Potable Water	Selected volumes of water, or dilutions thereof, are filtered through acetate membranes. Membranes are transferred to membrane lauryl sulphate agar and incubated at 30°C (recovery) followed by 37°C (growth). Following incubation yellow colonies (acid from lactose) are counted and multiplied by any dilution factor to give a presumptive result. Colonies are transferred to confirmatory media, lactose peptone water and incubated at 37°C. Acid and gas production by these colonies allow a calculation of the number of confirmed total coliforms. Samples should be analysed within 24 hours of sampling.
				Measured volumes of sample are filtered through a 0.45-µm pore size membrane filter which is then transferred to a selective culture medium. Incubation encourages the growth of Faecal Coliforms as colonies on the filter, while growth of other organisms is suppressed by the selective agent in the medium. Presumptive Faecal Coliforms colonies are recognised by their yellow colour, which results from fermentation of lactose to produce acid, in the presence of a pH indicator. Presumptive colonies may be subjected to further tests to confirm their identity if required. From knowledge of the volume of sample filtered and the number of colonies counted the concentration of Faecal Coliforms in the original sample can be calculated. Limit of detection is 1 organism in the volume analysed. (<1 cfu/100ml) Typical reporting volume cfu/100ml. Results are available 1-3 days after analysis. For Sediments / soil a known mass of sediment / soil is measured and a known quantity of diluent is added. This is then mixed and the resulting suspension analysed.

Method Title	Analyte	Accreditation	Matrix	Summary
NLS Coli Pot	Thermotolerant Coliforms Faecal Coliforms *E.coli*	UKAS	Potable Water	Members of the coliform group of bacteria, *Escherichia coli* and other Faecal coliforms, are the most commonly used micro-organisms as primary indicators of faecal pollution in waters of all types. The detection of such bacteria is taken as indicating the potential presence of intestinal pathogens. Samples should be analysed within 24 hours of sampling.
				Measured volumes of sample are filtered through a 0.45-µm pore size membrane filter which is then transferred to a selective culture medium. Incubation encourages the growth of Faecal coliforms / *E.coli* as colonies on the filter, while growth of other organisms is suppressed by the selective agent in the medium. Presumptive Faecal coliforms / *E.coli* colonies are recognised by their yellow colour, which results from fermentation of lactose to produce acid, in the presence of a pH indicator. Presumptive colonies may be subjected to further tests to confirm their identity if required. From knowledge of the volume of sample filtered and the number of colonies counted the concentration of Faecal coliforms / *E.coli* in the original sample can be calculated. Limit of detection is 1 organism in the volume analysed. (<1 cfu/100ml) Typical reporting volume cfu/100ml. Results are available 1-2 days after analysis. For Sediments / soil a known mass of sediment / soil is measured and a known quantity of diluent is added. This is then mixed and the resulting suspension analysed.

Method Title	Analyte	Accreditation	Matrix	Summary
NLS B Strep Pot	Enterococci FStrep Enterococcus faecalis Intestinal Enterococci (I.E.)	UKAS	Water, Soil, Sediment & Potable water	Faecal streptococci include a number of different species which occur in man and animals though in varying numbers. They are commonly used as indicators of faecal pollution in waters of all types. The main value of examination for faecal streptococci lies in assessing the significance of doubtful results from other tests. Since faecal streptococci are present in large numbers in faecal material (e.g. up to 10^6 per gram of fresh faeces) and can be detected in numbers as low as one organism in 100ml or more of water, they are currently a sensitive indicator available for all types of pollution. Samples should be analysed within 24 hours of sampling. Measured volumes of sample are filtered through a 0.45 µm pore size membrane filter which is then transferred to a selective culture medium. Incubation encourages the growth of faecal streptococci as colonies on the filter, while growth of other organisms is suppressed by the selective agents in the medium. Presumptive faecal streptococci colonies are recognised by their red, maroon or pink colour. Presumptive colonies may be subjected to further tests to confirm their identify if required. From knowledge of the volume of sample filtered and the number of colonies counted the concentration of faecal streptococci in the original sample can be calculated. Limit of detection is 1 organism in the volume analysed. (<1 cfu/100ml) Typical reporting volume cfu/100ml. Results are available 2-3 days after analysis. For Sediments / soil a known mass of sediment / soil is measured and a known quantity of diluent is added. This is then mixed and the resulting suspension analysed.

Method Title	Analyte	Accreditation	Matrix	Summary
NLS B PLATE COUNT	TVC Plate counts Heterotrophic Plate counts	UKAS	Potable water	A variety of micro-organisms, such as those associated with soil and vegetation, also occur naturally in ground and surface waters. Many of these organisms are usually able to survive for long periods in the environment and in the warmer months may multiply considerably. Counts of aerobic organisms which grow as colonies on plates of a nutrient agar under defined conditions thus provide an useful means of assessing the performance of water treatment processes. Such colony or plate counts can also provide a general indication of the bacterial content and hence the hygienic quality of water supplies, although the counts themselves have little direct health significance. In practice, changes in the pattern of colony counts of samples from a given supply are usually much more significant than the actual numerical count of any particular sample. A sudden marked change in the colony count of water in a supply may give forewarning of more serious pollution, whereas deviations in the expected seasonal trend may indicate longer term changes in the supply. Colony counts, if carried out regularly, are also of particular value when water is used for the large scale preparation of food and drink. Some bacteria can multiply within the distribution network by using nutrients derived from fixtures and fittings, which may colonise and cause production of biofilms Samples should be analysed within 24 hours of sampling.

Method Title	Analyte	Accreditation	Matrix	Summary
				Measured volumes of sample are analysed using the pour plate technique, using a non-selective medium. Knowing the volume of sample tested and any dilution factor, the samples original colony count can be calculated. Limit of detection is 1 organism in the volume analysed. (<1 cfu/ml). Typical reporting volume cfu/ml. Results may take up to 7 days to be available after analysis.
NLS B Somatic Phage	Somatic Phage Somatic Coliphage Coliphage	UKAS	Potable Waters	Bacteriophages are viruses that can only infect and replicate within Bacteria. In many cases, these are very specific relationships. These groups of Bacteriophages have been used as indirect indicators of Faecal pollution Somatic Coliphages that specifically infect Escherichia coli, have been used as an indirect indicator of Faecal pollution. Samples should be analysed within 48 hours of arrival in the laboratory.
				Measured volumes of sample and host bacteria are mixed gently with molten agar and then overlayed onto nutrient agar. Incubation encourages growth of the sensitive bacteria. The specific bacteriophage then destroys the bacterial cells by lysis, with the production of a plaque. From the knowledge of the volume and dilution of sample cultured and the number of plaques counted the concentration of bacteriophage can be calculated. Limit of detection plaque forming unit (pfu) in volume examined. The limit of detection may be affected depending on reporting volume and if concentration of the sample has taken place before analysis.

Method Title	Analyte	Accreditation	Matrix	Summary
				Results are available 1-2 days after analysis.

Method Summary for the Determination of pH, Electrical Conductivity and Turbidity in Waters by Automated Analysis

Determinand: pH (concentration of hydrogen ions)

Conductivity (μS/cm)

Turbidity (N.T.U.)

Matrix: Freshwater, effluent and saline

Instrumentation: Automated analyser system

Principle: pH and EC

The pH of a solution is defined by the equation:

$pH = -\log a_H$

where a_H is the activity of hydrogen ions in the solution. As the hydrogen ion activities cannot be determined experimentally the pH of a solution is determined by measuring the electromotive force (emf) of a cell containing the test solution and comparing it with the emf of a similar cell in which the test solution is replaced by a standard buffer solution.

Then:

$$\frac{pH(x) - pH(s)}{2.3026\ RT} = (E_s - E_x)F$$

Where pH(s) is the pH of the standard buffer solution

pH(x) is the pH of the unknown solution

Ex is the emf of the cell containing the test solution

Es is the emf of the standard buffer solution

R is the gas constant

T is the absolute temperature

F is the Faraday constant

The electrical conductivity of the sample is determined in a cell of known dimensions and will depend upon the concentration of ions. The unit of conductivity is siemen/metre although for convenience most measurements are reported in units of micro siemens/cm

(μS/cm). Conductivity is temperature dependent and therefore samples are quoted at 20°C.

Turbidity

Turbidity is an expression of the property by which suspended or colloidal matter scatters light thereby imparting opacity to the sample.

Light from a tungsten scource, scattered by matter in the sample, is measured at right angles to the incident beam. The intensity of the light scattered by the sample is compared with that measured for standard formazin suspensions and expressed as nephelometric turbidity units, NTU.

Range of Application: 0 – 14 pH units

1 – 100,000 μS/cm

1 – 100 NTU Turbidity

Range of application can be increased by dilution

Interferences: pH and EC

Gross suspended matter, oil or grease may cause interference by masking off part of the electrode surface.

Turbidity

Colour in the sample will cause absorption of light which may result in the measured values being low. Other errors may arise from dirty measuring cells, air bubbles and rapidly settling coarse particulates within the sample.

Within Laboratory Quality Control & Performance Criteria:

Total Error Target	< 20%
Bias Target	< 10%
Precision Targets	< 5% RSD

External Quality Control: Aquacheck/Quasimeme

Method Summary for the Determination of Suspended Solids by Filtration

Determinand: Organic and inorganic matter which can be removed from water by filtration

Matrix: Surface waters, groundwaters, sewages and discharges

Principle: Suspended matter is removed from a measured volume of sample by filtration under reduced pressure through a pre-washed, pre-weighed, pre-dried glass-fibre filter paper and determined gravimetrically after washing and drying at 105°C to constant weight

Range of Application: 50 mg for a 500 ml volume of sample. The range may be extended by reducing the aliquot of sample taken

Interferences: Oil and other organic liquids cause errors in the result. Samples which contain more than 1000mg/L dissolved solids may give high results

Within Laboratory Quality Control & Performance Criteria:

Total Error Target	< 20%
Bias Target	< 10%
Precision Targets	< 5% RSD

External Quality Control: Aquacheck/Quasimeme

Method Summary for the Determination of Colour in Waters by Spectrophotometer

Determinand:	Absorbance at a wavelength of 400nm after filtration
Matrix:	Surface waters, groundwaters, sewages and discharges
Instrumentation:	Perkin Elmer Lambda Spectrophotometer or equivalent system for measuring absorbance
Principle:	The sample is filtered through a cellulose acetate membrane of pore size and the absorbance of the filtrate is measured spectrophotometrically at 400nm. The result is expressed in Hazen units. The hazen standard unit is defined as the colour produced by 1mg/L platinum in the form of chloroplatinic acid in the presence of 2mg/L cobaltuous chloride hexahydrate
Range of Application:	Up to 50 Hazen Units colour. Tests have shown that the range is linear up to 500 Hazen Units. (Range may be extended by dilution)
Interferences:	None known

Within Laboratory Quality Control & Performance Criteria:

Precision – better than 5% RSD

Bias - better than 10% RSD

External Quality Control: Quasimeme, Aquacheck

Appendix C

RAW ANALYTICAL DATA:

- WORKS MONITORING PROGRAMME
- RECOVERY DATA: SPIKED WATER SAMPLES
- REPRODUCIBILITY: INTERLABORATORY COMPARISON OF qPCR data for NV

Table C:1 Works Monitoring Programme: Raw Data

VIRUSES IN WATER:TARGETED MONITORING USING THE LATEST METHODS

PARAMETER

ASSET C

DATE	SAMPLE POINT	COLOUR	CONDUCT	PH	TURBIDITY	SOLIDS	COLIF	ECOLI	ENTERO	TVC 22	TVC 37	F+PHAGE	CLOST	ADV	NV
		Hazen	uS/cm	pHunits	FTU	mg/ml	cfu/100ml	cfu/100ml	cfu/100ml	cfu/ml	cfu/ml	pfu/litre	CFU/100ml	gc/litre	gc/litre
07/06/2011	RAW	14	242	8	14.1	30.7	54	<10	<10	560	132	3		7588, ND, 695	ND, ND, ND
	CLARIF	<5	255	7.03	<1	<3	12	<1	<1	253	74	<2		ND,ND,ND	ND, ND, 212
	POST RGF	<5	302	7.81	<1	<3	<1	<1	<1	<1	<1	<2		ND,ND,1250	ND, ND, ND
	POST GAC	<5	296	7.4	<1	<1	<1	<1	<1	11	7	<2		ND,ND,ND	ND,ND,ND
02/08/2011	RAW	15	220	8.04	1.9	<3	1182	47	13	280	63	<10		1932, 762, 272	31, ND, ND
	CLARIF	<5	232	7.15	<1	<3	2	<1	<1	270	27	<2		ND,ND,ND	ND, ND, 21
	POST RGF	<5	283	7.71	<1	<3	<1	<1	<1	<1	<1	<2		ND, 1430, 532	ND,ND,ND
	POST GAC	<5	267	7.42	<1	<3	<1	<1	<1	11	3	<0.2		585, 713, ND	ND,ND,ND
08/11/2011	RAW	33	145	7.73	12.6	31.1	1200	100		19800	380	400	520	2382, 49, 1392	ND,ND,ND
	CLARIF	<5	159	6.86	1.19	<3	22	6		610	4	10	1	ND, ND, ND	ND, ND, 6
	POST RGF	<5	206	7.45	<1	<3	<1	<1		<1	<1	<10	<1	ND, ND, ND	ND, ND, 16
	POST GAC	<5	198	7.4	<1	<3	<1	<1		15	<1	<10	<1	ND, ND, ND	ND, ND, 233
17/01/2012	RAW	11	198	8.07	3.4	<3	88	63		540	45	6	18	83,67,56,ND,ND	ND,ND,ND
	CLARIF	<5	210	7.24	<1	<3	2	<1		203	16	<10	<1	ND,ND,ND	ND,ND,ND
	POST RGF	<5	249	7.67	1.6	<3	<1	<1		<1	<1	<10	<1	ND,ND,ND	ND,ND,ND
	POST GAC	<5	244	7.67	1.2	<3	<1	<1		23	<1	<10	<1	ND,ND,ND	ND,ND,ND
28/02/2012	RAW	8.2	208	8.08	4.4	5.07	27	18		2010	93	2	95	4310, 5415, 3884, 3140	55, ND, ND,832
	CLARIF	<5	219	7.15	1.3	<3	1	<1		210	31	<10	<10	ND,ND,ND,ND,ND	1417, ND, ND

	POST RGF	<5	251	7.67	<1	<3	<1	<1		<1	1	<10	<10	ND,ND,ND,ND,ND	ND, ND, ND
	POST GAC	<5	246	7.48	<1	<3	<1	<1		1450	41	<10	<10	ND,ND,ND,ND,ND	ND, ND, 221
ASSET E															
DATE	**SAMPLE POINT**	**COLOUR**	**CONDUCT**	**PH**	**TURBIDITY**	**SOLIDS**	**COLIF**	**ECOLI**	**ENTERO**	**TVC 22**	**TVC 37**	**F+PHAGE**	**CLOST**	**ADV**	**NV**
														gc/litre	**gc/litre**
15/06/2011	RAW	9.2	553	7.89	6.9	3610	410	198	113	800	310	<1		559, 873, 154	ND,ND,ND
	POSR CLARIF	<5	566	7.38	1.2	3.28	260	260	4	139	76	<2		360, ND, ND	ND,ND,ND
	POST RGF	<5	565	7.37	<1	25.1	64	46	1	247	29	2		ND, 276, ND	ND,ND,ND
	POST GAC	<5	568	7.5	<1	<3	<1	<1	<1	22	<1	<2		ND, 25, 206	ND,ND,ND
23/08/2011	RAW	34	424	7.93	4.7	6.35	1455	416	220	4600	1400	3		133, ND, 70	ND,ND,ND
	POST CLARIF	<5	501	6.74	2	10.2	100	80	6	860	150	<4		ND,ND,ND	ND,ND,ND
	POST RGF	<5	500	6.76	<1	<3	30	30	<1	239	34	<2		ND,ND,ND	ND,ND,ND
	POST GAC	<5	552	7.47	<1	<3	1	1	<1	170	1	<2		ND,ND,ND	ND,ND,ND
15/11/2011	RAW	21	478	8.19	5.8	4.77	790	790		9100	660	620	117	2685, 983, 293	ND,ND,ND
	POST GAC	<5	524	7.46	<1	<3	<1	<1		590	<1	<10	<1	3172, 251, 308	22, 2, ND
20/12/2011	RAW	26	482	7.98	8.8	7.4	3000	2000		16100	1010	269	306	ND, ND, ND	ND,ND,ND
	POST CLARIF	<5	486	7.11	1.3	8.35	45	45		6900	89	<10	50	ND, ND, ND	ND,22,ND
	POST RGF	5.9	485	7.23	<1	<3	10	7		1170	37	<10	1	ND, ND, 6	ND, ND, 130
	POST GAC	<5	495	7.21	<1	<3	1	<1		1600	5	<10	<1	ND, ND, ND	ND,ND,ND
06/03/2012	RAW	12	551	8.08	13.7	11.1	16200	1760		>30000	3200	33	235	6479, 7477, 7500	ND, 18, 2436
	POST CLARIF	<5	564	7.16	4	6.48	320	290		21300	950	<10	87	1753, 69, 64	37, 144, ND
	POST RGF	<5	564	7.19	<1	<3	26	7		>3000	67	<10	<1	ND,10,14	87, 51, 67
	POST GAC	<5	575	7.34	<1	<3	<1	<1		10	1	<10	<1	ND,ND,ND	18, 8, ND

ASSET T

DATE	SAMPLE POINT	COLOUR	CONDUCT	PH	TURBIDITY	SOLIDS	COLIF	ECOLI	ENTERO	TVC 22	TVC 37	F+PHAGE	CLOST	ADV	NV
														gc/litre	gc/litre
12/07/2011	RAW	<5	400	7.92	2.5	5.23	<10	<10	<10	1370	440	<1		2359, ND, 4007	ND,ND,ND
	POST DAF	<5	408	7.22	<1	3.58	2	<1	<1	10400	3700	<2		163, 5725, ND	ND,ND,ND
	POST RGF	<5	406	7.14	<1	<3	4	<1	<1	2340	1190	<2		ND, 595, ND	ND,ND,ND
13/09/2011	RAW	<5	432	8.42	<1	<3	171	45	<10	1610	860	<1		ND, 745, 154	ND, ND, ND
	POST DAF	<5	444	7.47	<1	<3	<1	<1	<1	4200	2300	<3		ND,ND,ND	ND, ND, ND
	POST RGF	<5	442	7.44	<1	<3	1	<1	<1	1210	790	<2		ND,ND,ND	ND, ND, ND
06/12/2011	RAW	<5	487	8.33	<1	<3	630	666		1150	65	340	45	56, 2465, ND	494,ND,ND
	POST DAF	<5	499	7.49	1.4	3.8	2	2		1590	266	<10	6	ND, ND, ND	ND,ND,ND
	POST RGF	<5	499	7.39	<1	<3	<1	<1		49	2	<10	<1	ND, ND, ND	ND,ND,ND
31/01/2012	RAW	6.5	524	8.37	2.2	<3	255	27		153	14	7	27	118,57,ND ND,ND,ND,ND	ND, ND, ND, ND, ND, 2336, 3504
	POST DAF	<5	534	7.43	1.3	<3	<1	<1		250	21	<10	9	ND,ND,ND	ND, 2480, ND
	POST RGF	<5	535	7.41	<1	<3	<1	<1		24	<1	<10	<1	ND,1300,1140	ND, ND, 36
27/03/2012	RAW	<5	477	8.25	2.1	3.6	<10	<10		660	16	<1	<10	ND,ND,ND	ND, ND, ND
	POST DAF	<5	493	7.49	<1	4.8	5	<1		>3000	20	<10	1	47,ND,ND	ND, ND, ND
	POST RGF	<5	493	7.42	3.1	<3	<1	<1		113	2	<10	<1	ND,ND,ND	ND, ND, 54

ASSET M

DATE	SAMPLE POINT	COLOUR	CONDUCT	PH	TURBIDITY	SOLIDS	COLIF	ECOLI	ENTERO	TVC 22	TVC 37	F+PHAGE	CLOST	ADV	NV
														gc/litre	gc/litre
19/07/2011	RAW	37	532	7.63	6.9	8.87	4560	512	<10	3000	770	27		779, 4420, 4285	ND,ND,ND
	POST CLAR	6.9	504	7.07	<1	<3	39	13	1	380	51	8		581, 1966, ND	ND,ND,ND
	POST OZON	<5	502	7.18	<1	<3	13	3	1	1780	145	<2		631, 345, ND	ND, ND, 278

	POST GAC	<5	491	7.02	<1	<3	2	1	<2	1770	1160	<2		1285, 488, ND	ND,ND,ND
06/09/2011	RAW	14	538	7.93	7.6	10.6	883	710	36	2200	350	42		780, 3839, 809	ND,ND,ND
	POST CLAR	5.1	540	7.43	<1	<3	4	1	1	630	25	12		ND,ND,ND	ND,ND,ND
	POST OZON	<5	541	7.46	<1	<3	8	6	1	720	26	28		ND,ND,ND	ND,ND,ND
	POST GAC	<5	541	7.39	<1	<3	3	<1	1	280	10	2		ND, ND, 1388	ND,ND,ND
13/12/2011	RAW	21	271	7.79	18.2	33.8	10636	1800		46000	2430	220	1120	21524, 40864, 36240	677,ND,ND
	POST CLAR	<5	276	6.95	<1	<3	<1	<1		320	<1	<10	<10	ND, ND, ND	ND,ND,ND
	POST OZON	<5	280	7.11	<1	<3	<1	<1		127	<1	<10	<10	ND, ND, ND	ND,ND,ND
	POST GAC	<5	263	6.64	<1	<3	<1	<1		140	14	10	<10	ND, ND, ND	ND,ND,ND
14/02/2012	RAW 1	14	456	7.92	6.7	7.42	4320	873		12200	990	71	360	10465, 8332, 6775, 8995	2290, 564, 3788, 2492
	RAW 2	13	456	7.92	6.3	7.52	2460	436		10900	740	80	342		
	POST CLAR 1	<5	452	7.24	<1	<3	13	4		1650	8	90	<1	ND,ND,ND,ND	ND, 18, 5
	POST CLAR 2	<5	452	7.23	1.1	3.47	13	5		310	4	110	<1		
	POST OZON 1	<5	453	7.22	<1	<3	18	7		380	4	70	<1	ND,ND,ND,ND	ND, 96, 27
	POST OZON 2	<5	453	7.24	<1	<3	13	4		230	1	40	<1		
	POST GAC 1	<5	450	7.11	<1	<3	9	1		260	5	<10	<1	ND,ND,ND,ND	ND, ND, ND
	POST GAC 2	<5	450	7.11	<1	<3	8	<1		340	2	10	<1		
20/03/2012	RAW	14	528	8.07	3.6	3.3	675	270		1330	250	3	189	3824, 9785, 7992	107, 19, 13
	POST CLAR	<5	537	7.44	<1	<3	24	6		300	4	40	<1	ND,ND,ND,ND	15, ND, ND
	POST OZON	<5	537	7.54	<1	<3	<1	<1		>3000	12	<10	<1	ND,ND,ND,ND	ND, ND, ND
	POST GAC	<5	531	7.33	<1	<3	<1	<1		370	<1	<10	<1	ND,ND,ND,ND	ND, ND, ND

Table C.2 Mean percentage recovery values for adenovirus and norovirus spikes.

	Data Group									
	C	E	M	T	Summer	Winter	Raw stage	First stage	Second	Final
Adenovirus										
mean	39.6	47.0	64.6	81.7	67.9	49.7	82.2	35.0	38.6	64.7
std dev	30.4	34.2	37.7	65.2	44.6	44.2	51.7	29.0	36.8	41.8
Norovirus										
mean	24.1	32.1	37.0	29.1	36.5	26.5	22.6	27.3	32.1	40.8
std dev	24.1	24.1	30.0	34.1	34.6	21.6	23.0	25.0	27.7	33.4
difference (Adv-HNV)										
	15.5	14.9	27.6	52.7	31.4	23.3	59.6	7.7	6.5	24.0

Appendix C:3 Reproducibility: qPCR data for the quantification of NV in a range of sample concentrates: RT-qPCR amplification details for each laboratory.

Starcross PCR assay:-

For both norovirus genogroup-specific assays, 3 aliquots of 5 µl RNA were tested in 25µl total volume with one-step reaction mix prepared using the One-Step RNA to Ct system (Life Technologies) (final concentrations of 1x Reaction Mix, 800 nM primers, and 50 nM probe. This master mix contains Rox at a set concentration from the manufacturer, and the enzyme concentrations used were as recommended by the manufacturer). Amplification was performed with the following cycling parameters; 48°C for 15 minutes, 95°C for 10 minutes, and then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute on a Mx3005P real-time PCR machine (Stratagene).

Cefas PCR assay:-

For both norovirus genogroup-specific assays, 2 aliquots of 5 µl RNA were tested in 25µl total volume with one-step reaction mix prepared using the RNA Ultrasense® one-step qRT-PCR system (Invitrogen) (final concentrations of 1x Reaction Mix, 500 nM forward and 900 nM reverse primers, and 250 nM probe, plus 0.5 µl Rox and 1.25 µl Enzyme Mix per reaction). For mengo virus QC material, two aliquots of 5 µl cDNA were used. Amplification was performed with the following cycling parameters; 55°C for 60 minutes, 95°C for 5 minutes, and then 45 cycles of 95°C for 15 seconds, 60°C for 1 minute and 65°C for 1 minute on a Mx3005P real-time PCR machine (Stratagene).

For both laboratories, the same primers, probes and acceptability criteria were applied: both laboratories used a four point standard curve, $5x10^4$ to $5x10^1$ in duplicate. The standard curve from each run had to meet the following: R-Sq >0.98, efficiency between 80 and 110%, and slope between -3.1 and -3.92.

Table C: 3 Estimates of norovirus concentrations from paired samples measured in the Starcross and the Cefas laboratories.

	log_{10}(norovirus)/unit volume	
Matrix/sample type	Starcross estimate	Cefas estimate
DNA standards	3.48	3.22
	3.48	3.14
	3.18	2.80
	3.18	2.75
	2.48	2.23
	2.48	2.21
	2.18	1.90
	2.18	1.91
	1.00	1.40
	2.00	2.33
	3.00	3.33
	4.00	4.27
	5.00	5.40
	6.00	6.40
	0.90	1.14
	2.04	2.04

	3.02	3.05
	4.00	3.98
RNA standards	4.70	3.87
	3.76	3.07
	5.86	5.17
	4.87	4.14
Faecal preps	8.79	9.05
	6.36	7.07
	6.43	7.47
Negative controls	0.00	0.00
	0.00	2.49
Positive controls	5.64	5.67
	6.36	6.27
Extracted RNA	5.29	6.07
	5.00	6.13
	4.43	5.87
	4.43	5.93
Raw waters	0.00	0.00
	0.00	0.00
	0.00	3.01
	0.00	0.00
	2.50	0.00
	4.25	5.53
	5.58	6.40
	4.88	6.84
	4.49	6.27
	4.86	0.00
	5.07	0.00
	5.79	1.67
Mid-process waters	0.00	0.00
	0.00	0.00
	4.50	6.33
	4.51	6.29
	6.10	7.29
	4.91	7.40
	5.50	7.35
	5.62	7.38
	5.90	7.49
	0.00	0.00
	0.00	0.00
	3.60	3.39
	2.51	2.41
	1.65	1.35
	0.70	0.24
	3.59	3.24
	2.44	2.10
	1.61	1.11

	0.59	0.42
	0.00	0.00
	0.00	0.00
	0.00	0.00
	0.00	0.00
	5.60	5.93
	3.10	5.24
	4.27	6.28
	4.29	5.83
	3.44	5.61
	5.77	6.80
	5.97	6.84
	4.14	5.66
	4.58	5.94
	4.36	6.84
	3.12	6.18
	0.00	5.23
	3.16	5.70
	3.54	5.51
	3.13	5.49
	4.81	6.95
	5.00	7.00
	0.00	0.00
	4.71	6.96
	2.50	4.60
	2.57	4.40
	3.86	4.60
	3.93	4.47
	4.64	5.07
Post-clarified water	5.36	0.00
	5.07	2.27
	5.71	4.33
	5.36	4.53
	5.50	4.60