EU FP VII PROJECT "VITAL"

STANDARD OPERATING PROCEDURE

SOP VITAL 004

Sampling and virus concentration from waters

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— WARNING —

Adenoviruses, noroviruses, and hepatitis A and E viruses are viral pathogens. All samples and controls shall be handled by trained staff in a laboratory with appropriate equipment. Staff must be fully vaccinated against Hepatitis A and poliovirus. Persons using this SOP must be familiar with normal 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.

AIM

To concentrate virus from a 10 litre volume of water (irrigation; processing; slaughterhouse effluent etc.) to a final volume of 10 ml of buffer.

PRINCIPLE

This protocol is based in the Standard Operating Procedure "Concentration method for fresh water" of the FP 6 Project VIROBATHE Contract 513648 (www.virobathe.org).

Ten litres of irrigation water including sample process controls are acidified and passed through a glass wool filter column using compressed air or a peristaltic pump. Virus particles adsorb to the glass wool and are later eluted by passing a protein eluant (beef extract) at high pH through the filter. The pH of the eluant is lowered to its isoelectric point (pH ~3.5) when a floc forms which contains proteins including virus. The floc is centrifuged and the deposit is resuspended in buffer.

EQUIPMENT

- pH Meter with temperature electrode or a thermometer
- Pressure vessel (to hold 10 l)
- Sterilised 10 l Plastic containers (preferably autoclaveable)
- Reinforced plastic tubing (8.0 × 12.7mm) 50-100cm length
- Connectors
- Filtration columns and stands
- Clamps
- Centrifuge
- Refrigerator
- Sterile graduated disposable pipettes (10ml; 5ml and 1ml)
- Sterile plastic bijoux
- Glass beakers (500ml)
- Centrifuge pots (300ml)
- Measuring cylinder (500ml)
- Glass Wool
- Plastic 100 ml measuring cylinder (adapted to pack glasswool into column)
- Disposable membrane filters and 20ml syringes (Luer-Lock) for secondary filtration

REAGENTS (SEE APPENDIX)

When working with chemicals, always wear suitable Personal Protective Equipment including a lab coat, disposable gloves, and protective goggles.

- Sodium thiosulphate (1.8% w/v) – if required
- Hydrochloric acid (1 N and 0.1 N)
- Sodium hydroxide (4% w/v = 1 N)
- Beef Extract (3% w/v) in glycine buffer (0.05 M)
- Deionised water
- Phosphate Buffered Saline (PBS)
- Freshly prepared hypochlorite (at least 1%), or equivalent disinfectant (e.g. Virkon)

PROCEDURE

1. Sampling

2. Collect 10 l of irrigation water in a sterile plastic bottle.
3. Label the plastic bottle for traceability, introducing, at least, the following details:
   Analyst
   Date of sampling
   Location
   Reference number (for traceability, use the same number for the rest of the analysis process)
4. Maintain the sample at 4ºC (max. 24 h).

2. Virus Concentration

2.1. Preparation of glass wool columns

1. Attach the lower end screw-cap to column, pack the column with 10 g glasswool and push down into the column (for example with the aid of 100 ml plastic cylinder and a universal bottle), to an approximate height of 6-8 cm.
2. Fix the packed column into the clamp stand in upright position.
3. Without using the upper screw-cap, slowly wash the column with about 50 ml of 1 N HCl, 50 ml of tap water, followed by 50 ml 4% NaOH and finally with tap water until the filtrate pH is neutral (at least 1 l). No pressure is needed, just pour reagents on top of the column and allow to drain through the column.
4. Prepare enough columns for the next day's samples. Cover the top of the column with aluminium foil.
5. When required for use, connect the column to the pressure vessel (see figure 1 below) or pump by attaching upper screw cap and tubing.

Figure 1 – Pressure vessel with glass wool column attached

2.2. Preparation of eluant

1. Check and record the calibration of the pH meter in the alkaline range and recalibrate if necessary.
2. For each water sample prepare 200 ml beef extract buffer at pH 9.5 (± 0.1), by addition of 4% sodium hydroxide. Record the pH of the beef extract.

2.3. Conditioning of Water

1. If the water contains chlorine, add 5 ml 1.8% sodium thiosulphate to the 10 l sample using a 10 ml sterile disposable pipette. Mix the water thoroughly and allow the water to stand (for 15 mins). This will ensure that any chlorine present in the water is neutralised.
2. Add 10 µl of the positive process control virus to the sample.
3. Check the calibration of the pH meter in the acid range and recalibrate if necessary.
4. On the worksheet record the pH of the water and its temperature (which should be between 15-22º C).

5. Lower the pH of the water to pH 3.5 ±0.1 by the addition of 1N HCl. Mix the water thoroughly by vigorous stirring while adding the acid.
6. Record the pH of the water after conditioning and the volume of 1N HCl used.
7. Transfer the water to the pressure vessel (if used).

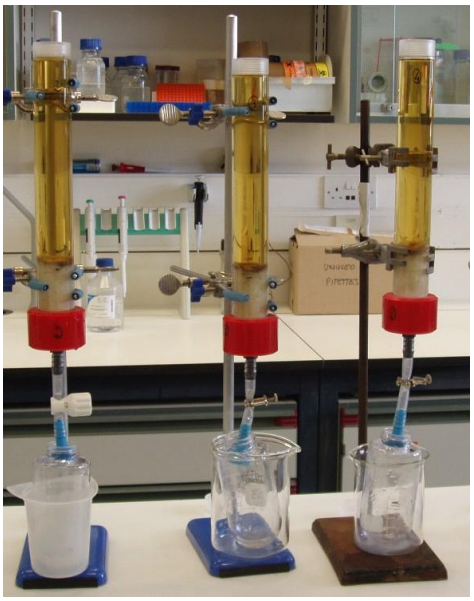
2.4. Filtration

Turn on the air line or pump slowly (do not exceed a rate of 1L/minute. Usually a pressure of <1 bar is sufficient). Record the time taken for the sample to pass through the glass wool filter.

2.5. Elution

1. When all the water has passed through the glass wool filter, close the outlet tubing of the column with a screw clamp.
2. Remove the top screw-cap of the column and slowly add 200 ml beef extract eluant at pH 9.5 (± 0.1) directly to the top of the glass wool. Open the clamp slightly to allow a small amount of the eluant to trickle out slowly into a centrifuge pot (supported inside a glass beaker) so that the whole of the glass wool filter is saturated then close the clamp again. Leave to stand for 10 minutes (see Figure2 below)
3. Open the clamp gently and allow the eluant to trickle out slowly into the centrifuge pot or glass beaker. Control the flow rate of the elution buffer using the clamp attached to the outlet tubing of the column.
4. Allow 25-30 minutes for the 200 ml of elution buffer to pass through the glass wool column. If the eluant passes through too fast the efficiency of elution will decrease. At the end, press down the glass wool with a clean universal bottle and the adapted plastic cylinder to squeeze out the last few drops from the filter.
5. Discard the used glass wool as appropriate for infectious material.

Figure 2 – Columns with eluant, before it passes through the filter



2.6. Flocculation

1. Lower the pH of the 200 ml eluted virus suspension very carefully to pH 3.5 (± 0.1) by adding 1 N HCl. Usually 1.5-2 ml is required. Use 0.1 N HCl when close to pH 3.5 and it may then be necessary to add the acid drop-by-drop. Lower the pH to the value that you have previously found to produce the maximum floc. This should be between pH 3.5 and pH 3.0. Stir the suspension continuously while adding acid.
   NOTE: The pH can not be lower than pH 3.0. If this happens, bring it back to pH 3.0 using NaOH.
   ATTENTION!!!: The pH electrode should be decontaminated very carefully in freshly prepared hypochlorite (at least 1%) or equivalent.
2. The floc should be visible as a darkening of the liquid with an increase in turbidity.

2.7. Refrigeration

Once flocculated store the suspension at 4ºC (± 3ºC) until the complete set of samples for that day are ready. Record the time of start of refrigeration

2.8. Cleaning of the Pressure Vessel (if used)

1. When cleaning, please use an appropriate virucidal disinfectant. Please remember to pass the disinfectant through the outlet and associated tubing, allowing enough contact time for the disinfectant to be effective. This will mean clamping the tubing after some of the disinfectant has passed through. Once enough time has elapsed, rinse thoroughly with distilled water (at least 3 times).

2.9. Centrifugation

1. Balance the pots on a balance by adding flocculated 3% beef extract buffer or use different pots with tap water for balancing. Do not attempt to balance pots by adding PBS or beef extract solution at neutral or alkaline pH. Mark the pots so the lower side can be identified after centrifugation.
2. Centrifuge at 7000 × *g* for 30 minutes. The floc usually forms a small pellet at the base of the centrifuge pot as well as depositing on the side of the pot.

2.10. Suspension of Pellet

1. Very gently pour off and discard the supernatant as appropriate for infectious material.
2. Add 8 ml PBS pH 7.2 (+/- 0.2) to each centrifugation pot.
3. Place the pots, with their lids on, at an angle or on their side so that buffer can come into contact with the deposit on the side of the pot. Leave for 30 mins at room temperature.

If the pellet is not dissolving then gently pipette the buffer up and down against the pellet. Do not pipette vigorously or create air bubbles. Measure the volume and add buffer so the total volume of suspension is 10 ml.

2.11. Post-process filtration

Secondary concentration may be necessary to remove bacteria or microscopic glass particles from the suspension. It improves the quality of the suspension without losing virus activity and is recommended especially where the concentrate is to be inoculated into cell cultures. The 4 ml for ICC-PCR must be put through a bacterial filter before freezing to remove particles and bacteria.

1. Prepare 1.5% w/v beef extract at pH 7.4. Pass approx 5 ml-10 ml through a disposable, single packed sterilized Sartorius Minisart 0.45 µm to saturate the filter and so prevent virus from attaching. If the concentrate contains a lot of particulates also saturate a Minisart 5 µm filter (this filter does not need to be sterile and can be bought in as a pack of 500) to act as a pre-filter and use in combination with the 0.45 µm filter.
2. Pass the 10 ml concentrate through the filters.
3. Store at –20°C.

APPENDIX

Hydrochloric Acid (1N)

34.4 ml concentrated hydrochloric acid (36% m/m)
400 ml deionised water

Measure 400 ml of deionised water in a measuring cylinder and then pour into a clean 500ml glass bottle. Using a 10ml disposable pipette tip add 34.4ml of concentrated hydrochloric acid. Label with the batch number and the expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	1 year
In-use	Room temperature	1 year

Hydrochloric Acid (0.1N)

10 ml 1 N hydrochloric acid
90 ml deionised water

Measure 90ml of deionised water in a measuring cylinder and add to a clean glass bottle. With a 10ml disposable pipette add 10ml of 1N hydrochloric acid. Label with the batch number and the expiry date. For storage and expiry dates see above.

Sodium Hydroxide (1N/4% w/v)

4 g sodium hydroxide
100 ml deionised water

Dissolve the sodium hydroxide in the deionised water in a sterile glass beaker. Once dissolved, dispense in 100ml volumes into clean glass bottles. Label with the batch number and expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	4 months
In-use	Room temperature	4 months

pH Buffers

pH buffers are supplied ready to use and a small volume should be aliquoted into a plastic universal when required. After use the aliquoted buffer and universal should be discarded.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
In-use	Room temperature	Day of use

Beef Extract (3% w/v) in Glycine Buffer (0.05M)

60 g Beef Extract
7.5 g Glycine
2 l deionised water

Add the beef extract and glycine to a clean 3 l conical flask. Add the water and stir with a magnetic stirrer to dissolve. This may take 2 hours. Dispense in 200 ml or 400 ml volumes. Autoclave to sterilise (example 121 ºC for 15 minutes). Store at room temperature.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	4 months
In-use	2 – 8 ºC	4 months

Before use the beef extract buffer must be quality controlled: place 10 ml portion autoclaved beef extract in a sterile plastic universal. Use 0.1 N HCl to lower the pH to 3.5 (± 0.1), monitor using the pH meter. Reduce the pH drop by drop until a visible floc forms. The floc must be visible at a pH less than pH 3 for the batch of beef extract to pass QC. Record the result and use this value for maximum flocculation.

Phosphate Buffered Saline (PBS)

10 Dulbecco's PBS tablets in 1 litre deionised water
or 2 Invitrogen or 2 Gibco PBS tablets in 1 litre deionised water

Using a magnetic stirrer, dissolve the PBS tablets in the deionised water. Once dissolved, aseptically dispense 10ml volumes into sterile universals.

If commercial PBS tablets are unavailable, please make in-house as set out below

In-house preparation:
Add 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 1000 ml molecular grade water to a bottle. Mix by stirring until the solids are dissolved. Adjust the pH to 7.3.

Sterilise all solutions according to local procedures e.g. autoclave at 121°C for 15 minutes. Check the sterility, of each batch made by plating out 100µl onto Nutrient Agar plates and record on the QC sheet. Label with the volume, batch number and the expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
In-house Stock	Room temperature	4 months
In-use	4°C (± 3°C)	4 months
In-media	4°C (± 3°C)	1 week

Sodium Thiosulphate pentahydrate (1.8% w/v)

9 g sodium thiosulphate.$5H_2O$
500 ml deionised water

Dissolve the sodium thiosulphate in a clean glass bottle. Label with the batch number and expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	1 year
In-use	2 – 8 ºC	1 year