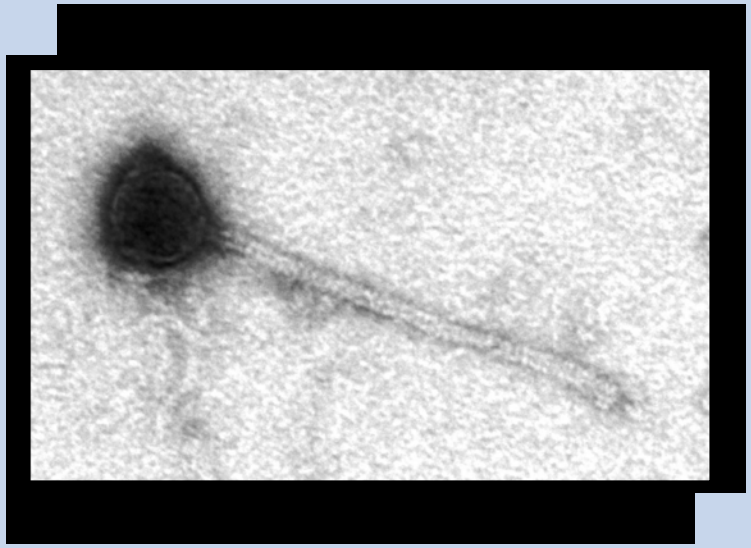
Somatic Coliphage Testing Manual

(Project SMaRT)



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MEDECINS SANS FRONTIERES
DOCTORS WITHOUT BORDERS

HIF Humanitarian innovation fund | elrha

University of Brighton

User Guidance

This illustrative manual was produced as part of Project SMaRT (Safer Management and Reliable Treatment of faecal sludge in humanitarian setting) and focusses on a method for the detection and enumeration of bacteriophages (Somatic coliphages) in faecal sludge. Bacteriophages are viruses capable of infecting bacteria and they tend to be more resistant than faecal indicator bacteria (such as E. coli) traditionally used to determine the efficacy of wastewater treatment processes. As such, somatic coliphages can give a better indication of the behaviour and removal of enteric viral pathogens.

The manual is principally designed for practitioners including non-governmental and public implementing organisations operating in low-resource settings, such as at field laboratories located at faecal sludge treatment facilities. However, the approach can also be used to test the performance of water treatment technologies.

Project SMaRT partners from the University of Brighton, Médecins Sans Frontières and BRAC, Bangladesh would like to thank the funder Elrha for their support and guidance, BluePhage® for provision of host bacteria, reference phage and growth media, and the not-for-profit design company Roots and Wings C.I.C for their creative input.

This manual is one of a series of three funded and supported by Elrha's Humanitarian Innovation Fund (HIF) programme, a grant making facility which improves outcomes for people affected by humanitarian crises by identifying, nurturing and sharing more effective, innovative and scalable solutions. Elrha's HIF is funded by aid from the UK Foreign, Commonwealth and Development Office (FCDO). Elrha is a global charity that finds solutions to complex humanitarian problems through research and innovation.
Visit www.elrha.org to find out more.

This manual has been developed to assist those working in emergency humanitarian settings where resources, including time, may be limited due to the urgent nature of the response. The manual is intended to support decision making and should complement, rather than substitute, sound professional judgement. The authors and publishers do not guarantee or accept legal liability of whatever nature arising from or connected to the content of this manual.

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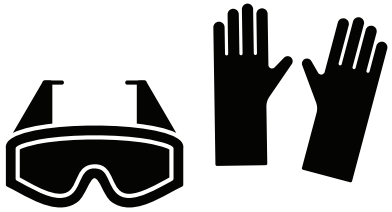
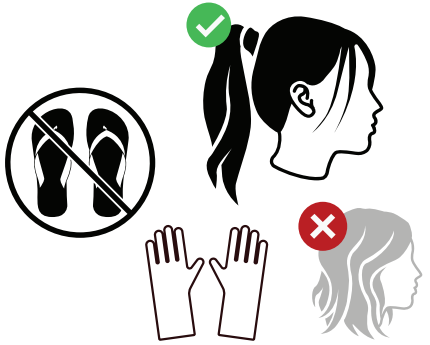
Protocol for the detection and enumeration of Somatic Coliphages

Background

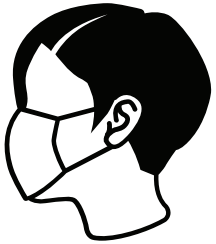
Detection and enumeration of Somatic Coliphages (SC) is performed using the double- agar layer technique involving Modified Scholten's (MS) media, which can either be purchased ready-made, or made following the recipe at the back of this manual. MS is a culture medium used in the enumeration of SC present in water, sediment and/or sludge samples. SC are detected and enumerated using Escherichia coli host strain (WG-5). Results of the test are expressed as plaque forming units (PFU) per 100mL, or 100g (wet weight) of sediment or sludge. All work should be carried out in an aseptic environment using the necessary Personal Protective Equipment (PPE), ideally next to a flame (convection source) to prevent cross-contamination. The following procedure is based on ISO 10705-2 (Water quality - Detection and enumeration of bacteriophages - Part 2: enumeration of somatic coliphages).



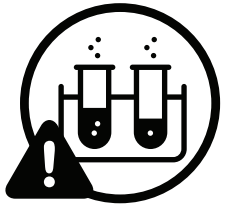
Labcoat, safety glasses, gloves should be used when handling the host bacteria (E. coli), and when filtering and handling samples



Thermally insulated gloves should be used when handling melted (molten) agar. Shoes must be closed toe (no sandals) and long hair should be tied back



Facemask should also be used when preparing and weighing out reagents (e.g. powdered agar media)

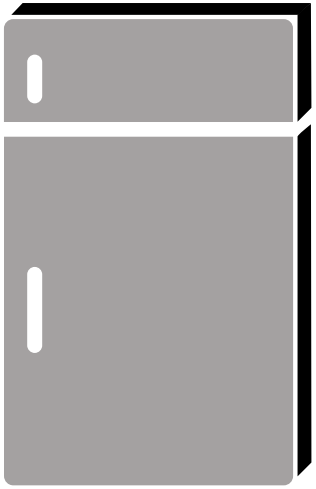


Particular care must be taken if using glass Petri dishes or test tubes

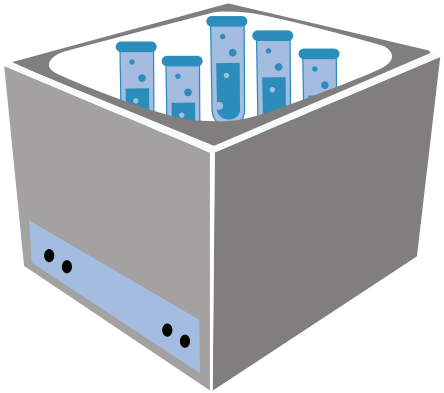
Apparatus

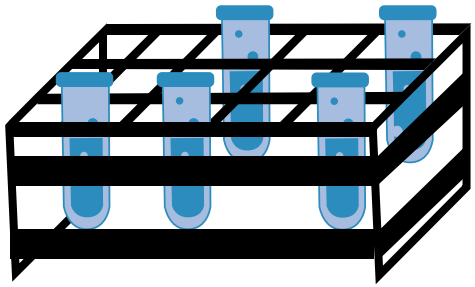
Equipment and Media (suggested amount)

- Refrigerator ideally with small freezer compartment – domestic one fine (x1)



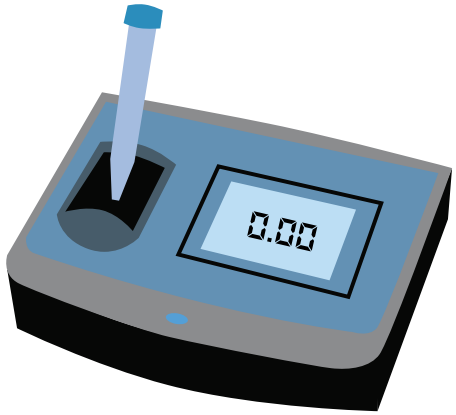
- Water bath capable of maintaining temperatures of 50-55°C (x1) & test tube racks (x2)





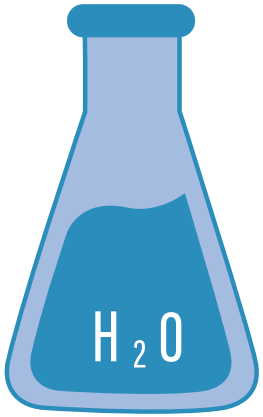
- Vortexer e.g. WhirlimixerTM (Fisher Scientific) or equivalent (x1)

- Spectrophotometer (capable of reading at 600nm) (x1) and cuvette (glass vial(s)),

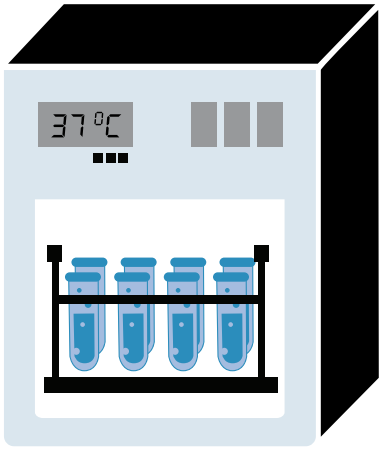


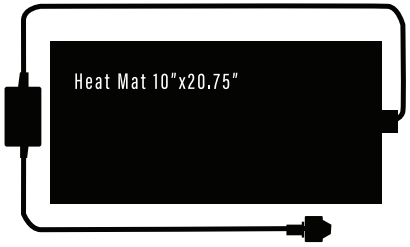
- Sterile Schott bottles (e.g. 10 x 100mL or 200mL, 2 x 500mL and 2 x 1L) or similar autoclavable vessels with lids to prepare media (agar, semi-solid agar and broth) and to grow the host bacteria (strain WG-5)

- Source of distilled water for preparing media (MS broth, agar).

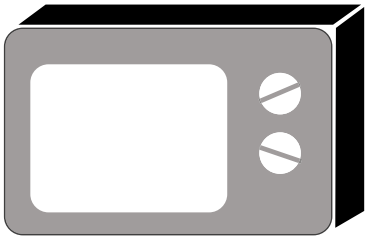


- Incubator or heated mat capable of being set at 37°C (x1)



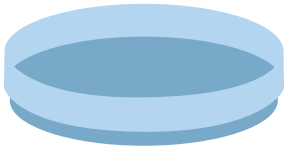


- Microwave for heating media and for melting semi-solid agar – domestic one fine (x 1)

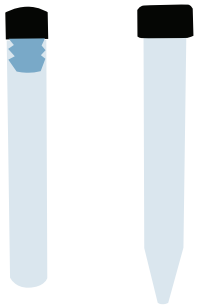


- Plastic measuring jugs 1 L (x4) and plastic stirring rods for mixing media (x2)

- Petri dishes 90mm diameter (e.g. single-use plastic) though glass can be used (x500)

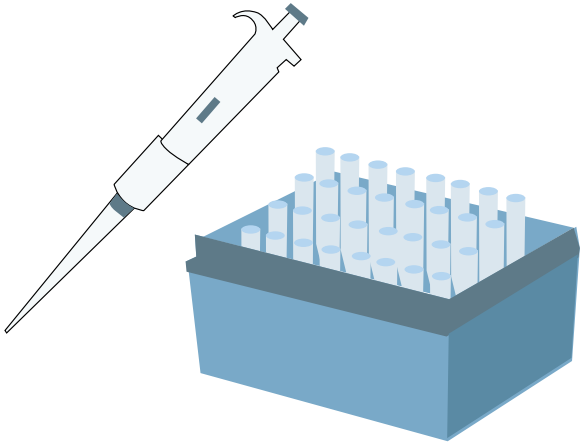


- Test tubes (10-12mL) with lids ideally disposable plastic (e.g. SterillinTM or Falcon tubes) though glass can be used (x 500)

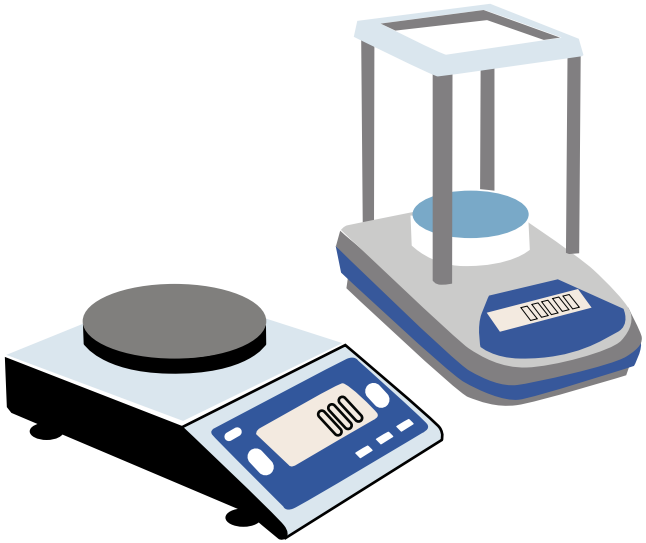


- Plastic 10mL syringes – push fit or luer-lock fitting and 0.22µm polyvinylidene difluoride membrane syringe filter units (e.g. PVDS or PES) x500

- Pipettes (1 or 2 x 1mL and 1 or two x 5mL/10mL capacity (plus sterile tips, with filters if possible))



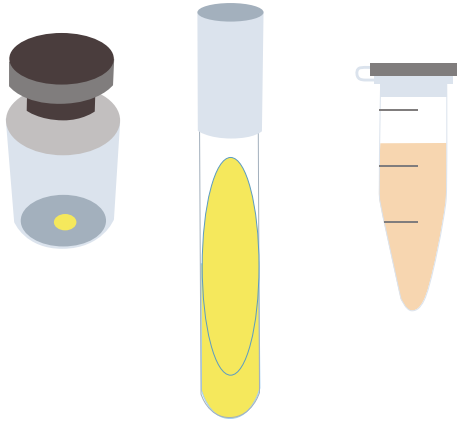
- Analytical laboratory balance (x 1) and weighing boats (x50)



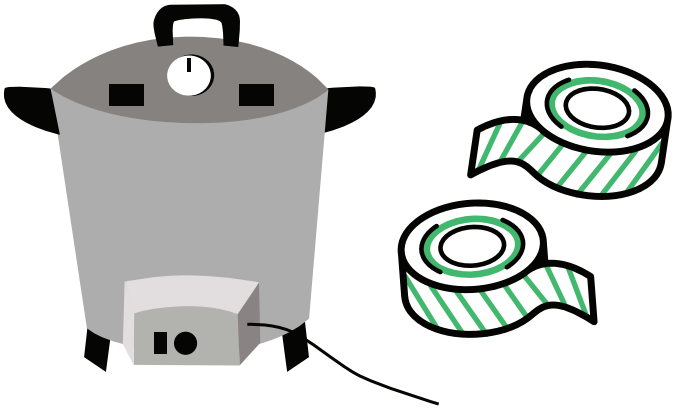
- Modified Scholtens' broth (MSB) and Modified Scholtens agar (MSA)

- Agar Bacteriological (Agar No.1)

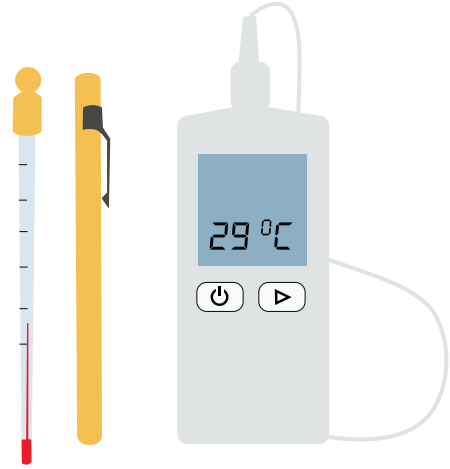
- Host bacteria (*E. coli*) strain WG-5 - either as freeze dried tablets stored at -20°C, agar slants stored at 4°C or vials stored at -20 or -80°C



- Autoclave capable of reaching 121°C for 15 mins (x1) and autoclave tape (x5)



- Thermometer for monitoring water bath and incubator temperatures (Digital or glass, alcohol type) (x2)



Media preparation

Step 1. To produce Modified Scholtens Broth (MSB), weigh out 29.4g of MSB powder* either pre-made (e.g. bluephage® Product code: BP1637), or following the recipe at the rear of this manual) into a weighing boat and dissolve (by gently stirring) in 1 litre of distilled H2O (in a 1L measuring jug).

Step 2. Pour half (500mL) of MSB solution into either a 500mL Schott bottle (or divide into 5 x 100mL bottles), label 'MSB' and autoclave at 121 °C for 15 mins. Once cooled keep refrigerated until use, or for up to 6 months.

Step 3. To produce Modified Scholtens Agar semi-solid (MSAss), weigh out 4.0g of Agar (Bacteriological) and add to the other 500mL of MSB solution in the 1L measuring jug. Carefully heat to boiling in microwave until the agar powder is fully dissolved (the solution becomes clear). Careful that the contents of the jug do not boil over! This solution (MSB + Agar = MSAss) can now be decanted into 5 x 100mL Schott bottles, labelled 'MSAss' and autoclaved at 121 °C for 15 mins. Once cooled keep refrigerated until use, or for up to 6 months.

Step 4. To produce Modified Scholtens Agar (MSA), weigh out 44.35g MSA powder*, either pre-made (e.g. bluephage® Product code: BP1638), or following the recipe at the rear of this manual) into a weighing boat and carefully pour the contents of the weighing boat into an empty 1L Schott bottle. Measure out 1 litre of distilled H2O and carefully pour it into the Schott bottle containing the powder. Label the bottle 'MSA' and autoclave at 121 °C for 15 mins. The autoclaved bottle can be left to cool down to a safe temperature (e.g. 50-60°C) or ideally placed in an incubator set at 55°C. Once cool enough, add 6mL per L of CaCl2 solution (14.6g per 100mL), and mix gently, then pour the molten MSA into 90mm Petri dishes (just enough to cover the plate). Once the plates have cooled and the MSA set then they can be bagged up and stored (agar side up) in the refrigerator until use, or for up to 4 months.

Note: It is important to always maintain aseptic conditions when pouring the prepared media into Petri dishes, utilizing an alcohol lamp, Bunsen burner or laminar flow cabinet if available.

* Premade MSB/MSA Powder storage: Keep container tightly closed, away from bright light in a cool dry place (4°C to 30°C). Shelf-life: 5 years.

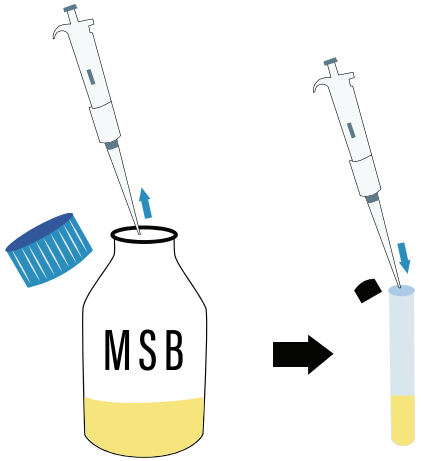
Note: The method still works without the addition of the CaCl2 solution, only the plaques may be less defined.

Detecting and enumerating Somatic Coliphages (SC)

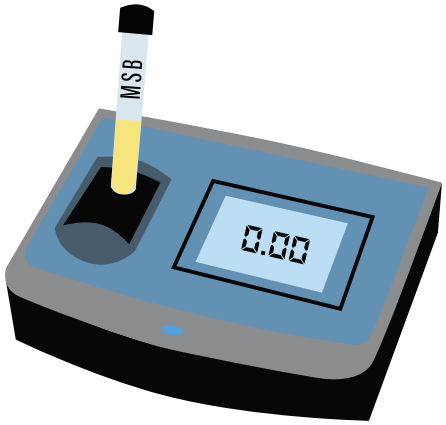
Equipment and Media (suggested amount)

Step 1. Remove Schott Bottle containing 100mL of MSB from fridge and allow to warm to room temperature.

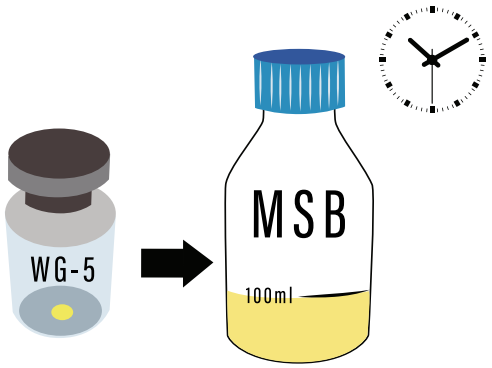
Step 2. Aseptically remove a small volume (e.g. 2mL or 10mL) of the MSB using a pipette and add to and a cuvette or glass vial. This is the Blank.



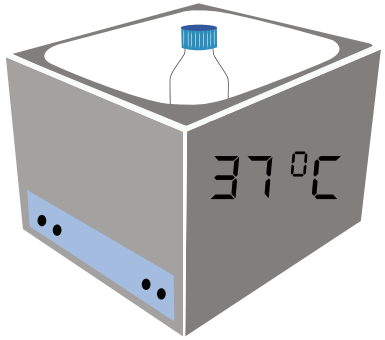
Step 3. Put the Blank (cuvette or glass vial containing the MSB) into the spectrophotometer set to read at 600nm and zero.

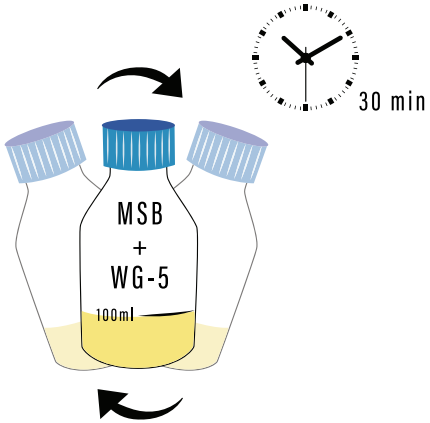


Step 4. Add *E. coli* host strain WG-5 (either as freeze-dried tablet, or from agar slant, or from a defrosted vial) to the Schott bottle containing 100mL of MSB, label WG-5 and note the time the host strain was added.

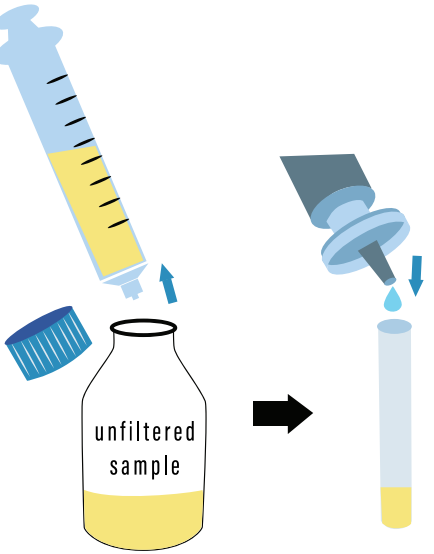


Step 5. Place the Schott bottle containing WG-5 host into a water bath set to 37°C and leave the host strain to grow. Unless a shaking water bath is available, gently swirl the bottle and contents from time to time (e.g. every 30 mins) during the incubation period.





Step 6. Meanwhile filter-sterilise samples to be tested (to remove bacteria, fungi etc) through 0.22µm pore size syringe driven filter into sterile test tubes. You can do this prior to analysis (e.g. the day before, provided you store the samples in the fridge at approx. 4°C).



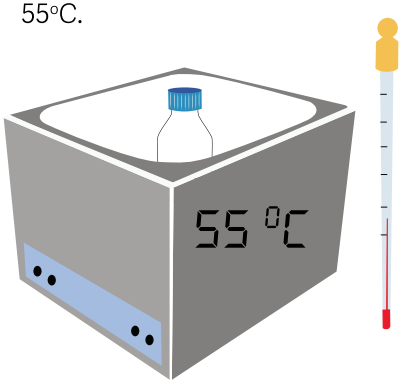
Step 7. After approx 1-1.5 hr remove the host WG-5 from the waterbath and test the optical density (OD) by carefully removing a small volume (e.g. 2mL or 10mL) using a pipette and adding to a cuvette, or glass vial, before inserting into the spectrophotometer set at 600nm. Make a note of the OD and time. Continue to monitor (every 30 mins) until it reaches an optical density of approximately 0.33 (but up to 0.50 will still be fine).

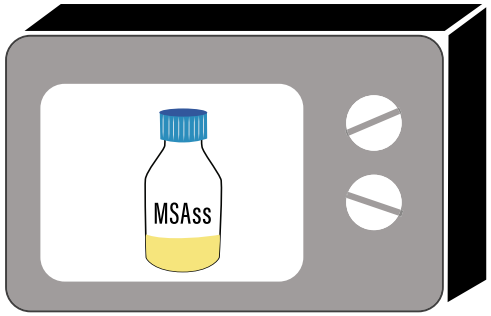
Note: When filtering samples and performing phage detection and enumeration, it is advisable to to keep the ambient room temperature stable (where possible).

Note: WG-5 can take between up to 2-3 hours to grow (depending on the starting density of the host and volume of MSB used) so it's best to get this growing as early as possible.

Step 8. Once the optical density is reached, place the host (WG-5) in fridge, or on melting ice, or on a cooler pack to stop the culture from growing further (use within the same day).

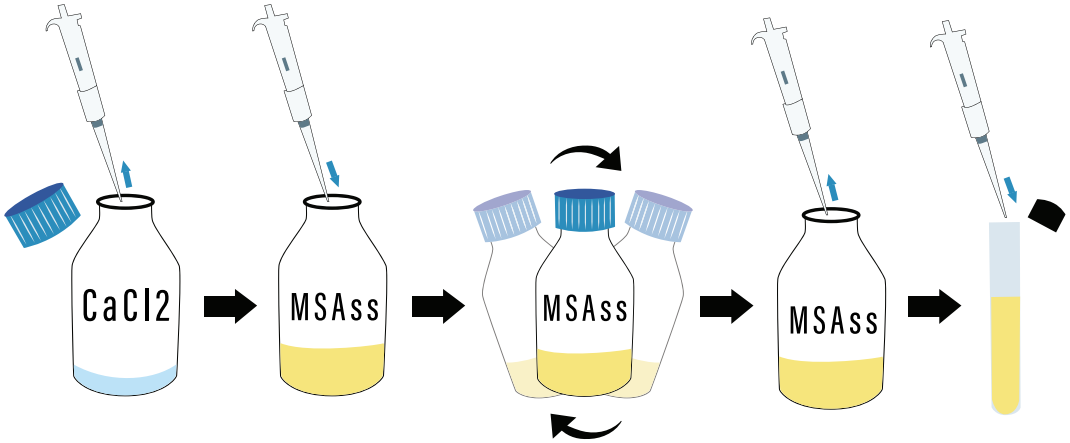
Step 9. Remove a 100mL Schott bottle containing MSAss from fridge and melt the in the microwave on a low setting (to avoid the media boiling over), or place the Schott bottle in a beaker of boiling water and heat. Use heat-proof gloves to transfer the molten MSAss to a pre-warmed water bath set to 55°C.



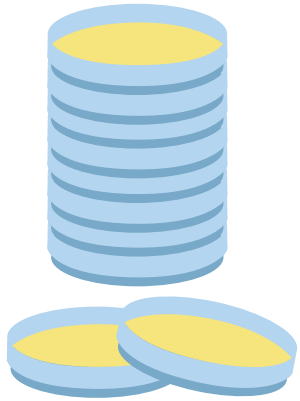


Note: Temperature settings on the water baths don't tend to be very accurate so best to test the water temperature using thermometer prior to placing semi-solid agar in the bath, as the agar can set prematurely if in contact with a chilled sample.

Step 10. Once the MSAss has cooled a little carefully pipette 0.6mL of CaCl2 solution (this can be made in advance as per recipe at back of protocol) to 100mL of MSAss (this helps to make the zones of lysis or 'plaques' clearer). Gently, swirl before pipetting 2.5mL MSAss into test tubes (with lids) and place them into a test tube rack in the water bath at 55°C to keep from setting. Calculate how many test tubes of MSAss you will need (which depends on how many samples you are testing).



Step 11. Remove Petri dishes containing MSA from fridge a few hours prior to use (to allow to them to warm to room temperature and reduce condensation).



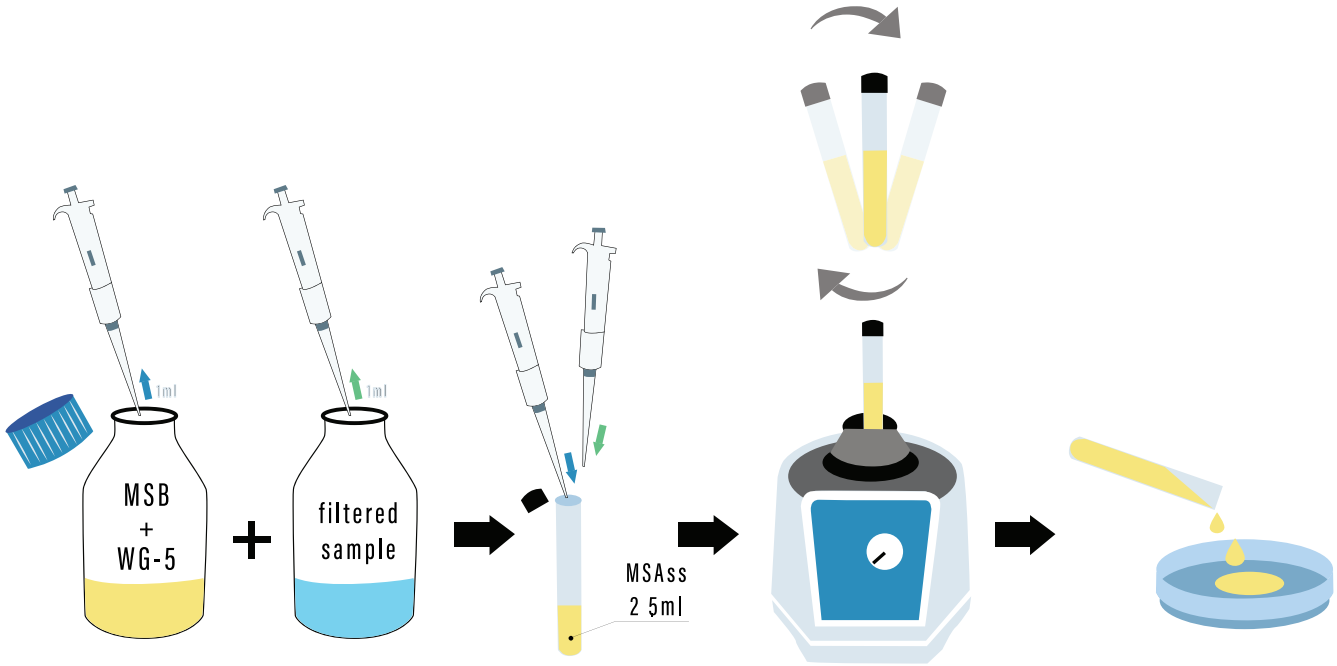
Step 12. Label the Petri dishes (side containing agar, not lid) with sample information (e.g. origin, volume and/or dilution tested) 90 mm Petri plate containing MSA.

Note: You can also use codes to simplify labelling.

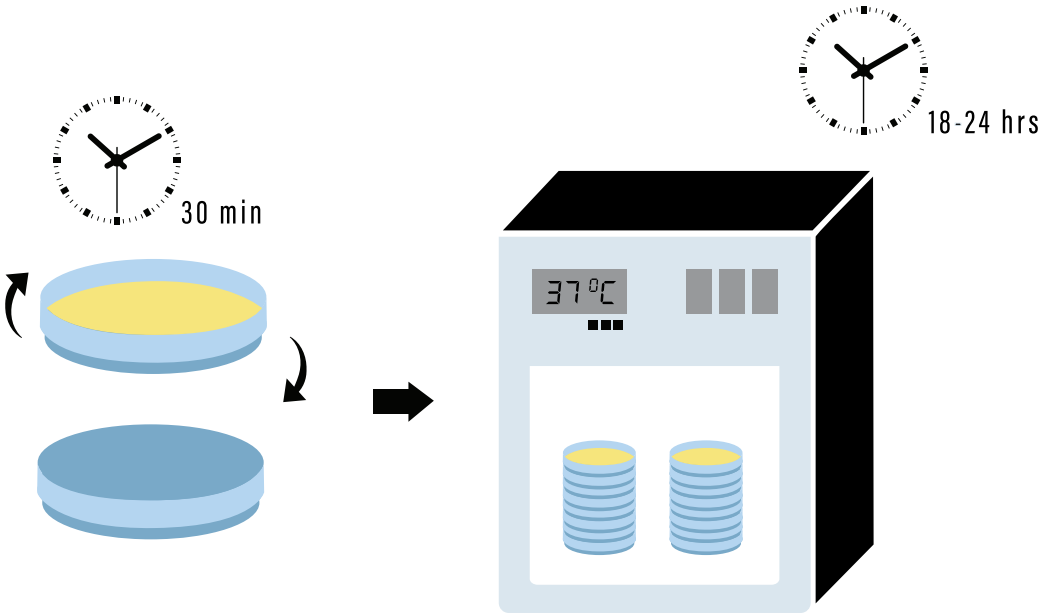
Example of a labeled Petri dish base

Step 13. Using sterile tips, carefully pipette 1mL of the host strain (WG-5) and 1mL of your filtered sample into test tube containing 2.5mL of MSAss (be sure to use a fresh tip for each sample and particularly when handling the host WG-5 and the sample to avoid cross-contamination). Mix briefly on a vortexer (avoiding bubbles) and pour onto a the surface of the labelled Petri dish.

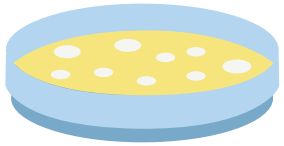
Note: It is very important that the MSAss mixture is not outside the water bath too long before pouring, as it can set prematurely



Step 14. Leave the Petri plates for at least 30 mins until they are set and invert before incubating for 18-24 hrs at 37°C.



Step 15. After 18-24 hours count the number of plaque-forming units (PFU). Results are generally expressed per as PFU per 100mL, or 100g (wet weight) of soil or sludge. Somatic coliphage tend to produce clear plaques which can differ significantly in size (diameter).



Note: If fungal/bacterial cross-contamination occurs on the Petri plates during incubation, then antibiotics (e.g. nalidixic acid can be added to the molten MSAss media to give a final concentration of 250 µg/ml). Nalidixic acid is heat-stable and can either be added from a filter-sterilized solution after melting of MSAss, or added before autoclaving.

Note: Plaques are visible after just 4 hrs incubation and can give an indication of the likely count after 18-24 hrs incubation. This is particularly useful in samples which are likely to contain high numbers of phages (e.g. raw wastewater).

Note: It is always important to run a procedural blank e.g. distilled H2O, or boiled water (cooled), Phosphate Buffered Saline (PBS) or 1⁄4 strength Ringers solution in place of the sample. Reference phage e.g. ϕX174 or other phage active against WG-5 should also be used where possible as a positive control.

Note: If using the ϕX174 (supplied by Bluephage®) with 0.5 mL of Phosphate Buffered Saline (PBS) and after gently shaking add 1 mL more. Allow 10 min to complete reconstitution. The final concentration of the suspension will be 20-90 PFU/mL, depending on the batch, and will be marked on the vial. Discard the reference control if the mean number of pfu/mL is not into the range of values indicated on the label.

Appendix 1

Recipe for preparing Modified Scholtens Media

(if pre-made media is not available)

Modified Scholtens Broth (MSB)

Peptone or Special Peptone	10g
Yeast extract	3g
Meat extract (e.g. LAB-LEMCO)	12g
NaCl	3g
Na_2CO_3	0.75g
$MgCl_2$ (Anhydrous)	0.6g
Distilled H_2O	1000mL

Modified Scholtens Agar (MSA)

Prepare as for **MSB** but add Agar (bacteriological)	16g (per litre)

Modified Scholtens semi-solid Agar (MSAss)

Prepare as for **MSB** but add Agar (bacteriological)	8g (per litre)

After autoclaving add 0.6mL (600µL) of sterile $CaCl_2$ solution (14.6g per 100mL) per 100mL of **MSAss** and 6mL per litre of **MSA.**

Note: Do not add $CaCl_2$ solution to MSB as it alters the optical density of the broth!

Note: If testing using freeze-dried *E. coli* (WG-5) tablets (supplied by Bluephage®), prewarm 10 mL of MSB in a sterile in Schott bottle at 37°C, before aseptically adding the tablet to the bottle. Allow 10 min for rehydration. Then incubate at 37°C for 120-150 min in a shaking water bath (if available) or incubator. After the incubation time, quickly cool the culture by placing it in a refrigerator or on melting ice. At this time, the bacterial culture should present a concentration of approx. 8-9 log Colony Forming Units (CFU)*/mL . Use the inoculum culture within the same working day.

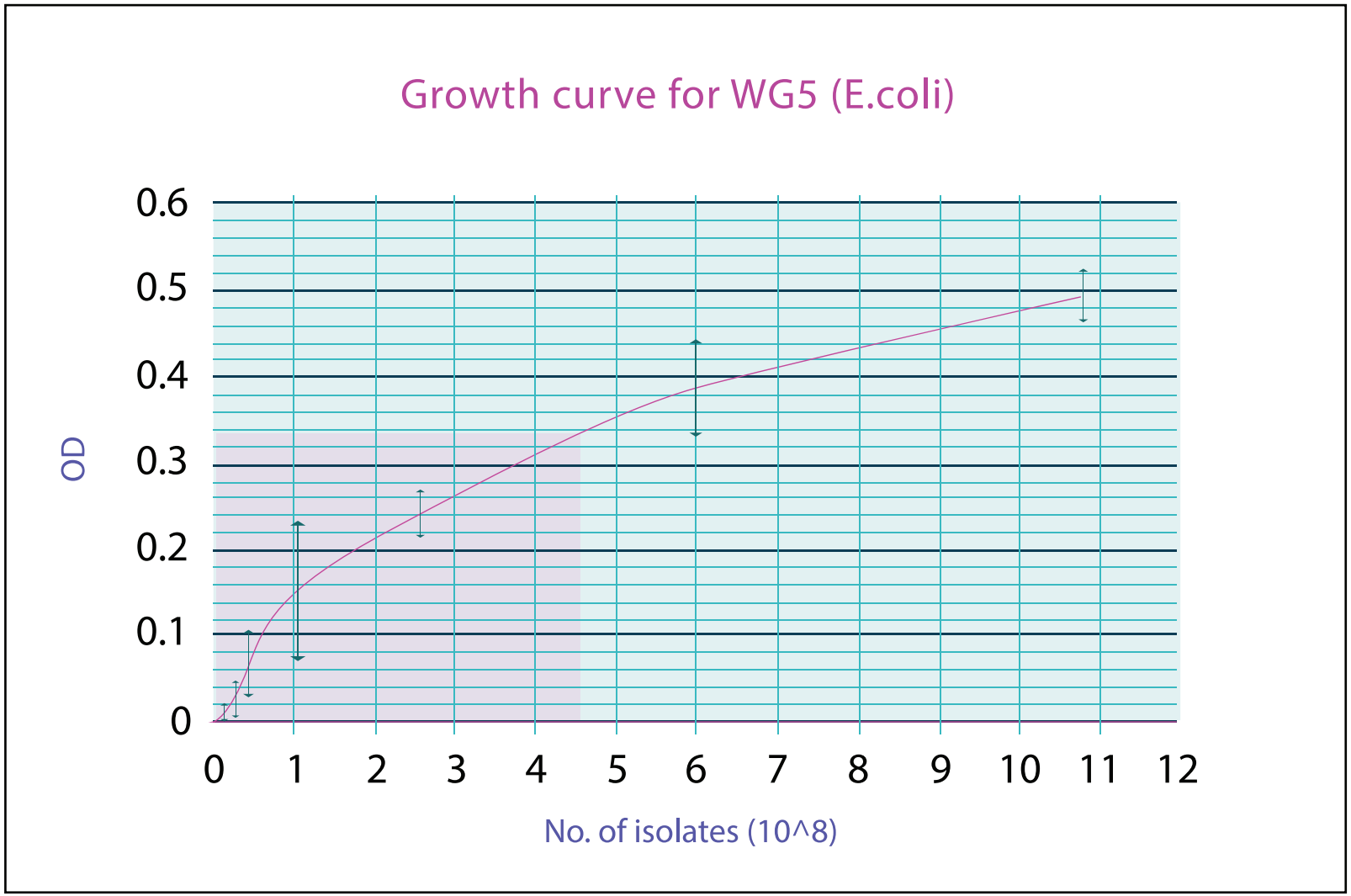


Figure A.1. Growth curve used to establish relationship between the optical density (0.33 at 600nm) and no. of isolates (colonies) needed to ensure a confluent lawn of host (WG-5), needed to clearly visualise plaques (zones of lysis).

References

Anon. (2001) [b]. ISO 10705-2, Water quality - Detection and enumeration of bacteriophages - Part 2: enumeration of somatic coliphages. International Organisation for Standardisation, Geneva, Switzerland.

'Further information about the Premade MSB, MSA media and about freeze-dried host WG-5 and reference phage can be found at the Bluephage® site (**https://bluephage.com/iso-kits/**)'

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