Tube-free Agar Overlays: rapid plaque assays with fewer steps and materials

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ABSTRACT

This protocol simplifies current approaches for agar overlay plaque assays by eliminating the use of tubes for premixing of agar, hosts, and viruses, in favor of pipetting each of these directly onto the bottom agar. The benefits of this approach include simplified experimental set-up, and reductions in preparation and clean-up times and material requirements. In addition, by eliminating tube-based steps, the duration of exposure of cells and viruses to heat is reduced and the need for potentially damaging vortexing or agitation of virus-host mixtures is also eliminated. We note that Hershey et al. [1] mentioned the possibility of such an approach in their 1943 description of the tube-based overlay procedure, stating: “A further slight improvement may be made by mixing the sample directly on the phage with only 3 ml 0.7 per cent agar, but the mixing is difficult.” By using a lower percentage agar, which is advantageous for other reasons (see Additional Information section in the associated publication), we find that this approach works very well with as low as 2 ml of top agar.

Using this streamlined, tube-free, plating method, >45 samples can readily be plated per hour from a common or pre-prepared virus stock, without the need for individual tubes of molten agar or multiple transfers of bacteria and virus. When deployed for isolation of novel viruses, or quantification of environmental viruses, this translates to a potential for screening hundreds of potential host strains per day using a single bottle of molten agar rather than hundreds of tubes. When used for routine bench assays, a single bottle of top agar can be used over multiple days by simply re-microwaving.

[1] A. Hershey, G. Kalmanson, J. Bronfenbrenner "Quantitative methods in the study of the phage-antiphage reaction" J. Immunol., 46 (1943), pp. 267-279

EXTERNAL LINK

https://doi.org/10.1016/j.mex.2018.01.007

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Kauffman, K. M. & Polz, M. F. Streamlining standard bacteriophage methods for higher throughput. MethodsX 5, 159–172 (2018). https://doi.org/10.1016/j.mex.2018.01.007 - This protocols.io protocol has been adapted from the MethodsX article, which is published under the Creative Commons Attribution License (CC BY).

PDF

Kauffman and Polz - 2018 - Streamlining standard bacteriophage methods for hi.pdf

PROTOCOL STATUS

Working

GUIDELINES

Please also refer to the original publication for a **"Microreview of additional considerations relevant to agar overlays"**, which highlights methodological aspects that are known or expected to impact the nature, number, and diversity of viruses recovered using agar overlay approaches.

MATERIALS TEXT

See "Guidelines & Warnings" section for materials to have ready before start.

SAFETY WARNINGS

Use caution when microwaving top agar, ensure that the bottle cap is unscrewed to allow venting, remove from the microwave with heat-safe gloves, and do not immediately swirl as this may cause sudden boil-over.

BEFORE STARTING

Materials to prepare in advance for agar overlay approaches

Host culture: This procedure works well with 100 ul of overnight host culture for each standard size (100 mm) petri dish.

Virus material:

- For ***Isolation of Viruses*** the appropriate stock material can include any potential virus source, for example: an iron-chloride flocculate of filtered seawater, resuspended in oxalate solution (John et al. 2011 [2], Poulos et al. 2018 [3]); a PEG-precipitate of sewage supernatant; or soil or stool resuspended in buffer and then pelleted or filtered to remove cells. The total volume needed per plate depends on the concentration of viruses in the stock material but the agar overlay procedure can accommodate up to several hundred microliters of material; at higher volumes of stock material (greater ~ 100 ul) it is recommended that the volume of host and top agar be increased proportionately, up to 3 ml top agar.
- For ***Quantification of Virus Titer by Direct Plating*** the appropriate stock material is a 10-fold dilution series of the virus stock in buffer or media, with a recommended plating volume of ≥10 ul per plate.

Bottom agar plates: Prepare media containing 1.0% agar ('bottom agar') in a glass bottle or flask with a stirbar and sterilize, pour 25 ml per standard size petri dishes, and allow to solidify.

Top agar bottle: Prepare media containing 0.3% agar ('0.3% top agar') in a glass bottle with a PTFE-coated stirbar and sterilize; though each plate will require only 2ml of top agar, volumes of up to 500 ml can be prepared and re-used across multiple days of plating.

[2] John, S.G., Mendez, C.B., Deng, L., Poulos, B., Kauffman, A.K.M., Kern, S., Brum, J., Polz, M.F., Boyle, E.A., and Sullivan, M.B. (2011). A simple and efficient method for concentration of ocean viruses by chemical flocculation. Environmental Microbiology Reports 3, 195–202.

[3] Poulos, B.T., John, S.G., and Sullivan, M.B. (2018). Iron Chloride Flocculation of Bacteriophages from Seawater. In Bacteriophages, (Humana Press, New York, NY), pp. 49–57.

I. Preparation of the top agar in beaker-waterbath

1 **Prepare a beaker-waterbath:** Place bottle of top agar into a glass beaker and add water to the beaker up to the level of the top agar in the bottle; for example, a 500 ml glass bottle into a 1l beaker.

2 **Melt the top agar:** Place the beaker-waterbath with the top agar bottle into a microwave and cook without boil-over until thoroughly melted.

NOTE

It is exceedingly important to achieve a 'smooth melt' of the top agar to ensure that plaques will form and be visible. Media will differ in the time it takes to achieve a 'smooth melt', however a general approach is as follows: start by melting the top agar for several 5-minute cycles at low % power, once the top agar appears nearly completely melted increase the % power and cook until the top agar comes to a boil, observe the top agar during high power cooking to prevent boil-over, bring to a boil 3 times.

SAFETY INFORMATION

(1) Ensure that the lid of the top agar bottle is slightly loose to allow for venting during microwaving.

SAFETY INFORMATION

(2) Remove top agar bottle from the microwave with heat-safe gloves.

SAFETY INFORMATION

(3) Do not swirl the top agar bottle while removing from microwave as this may cause sudden boil-over resulting in burns.

3

Equilibrate the top agar to 50-52°C: Place the beaker-waterbath containing the bottle of molten top agar and stirbar onto a hot plate and activate gentle stirring, place a thermometer into the beaker-waterbath and leave it there to wait for equilibration to 50-52°C (Figure 1).

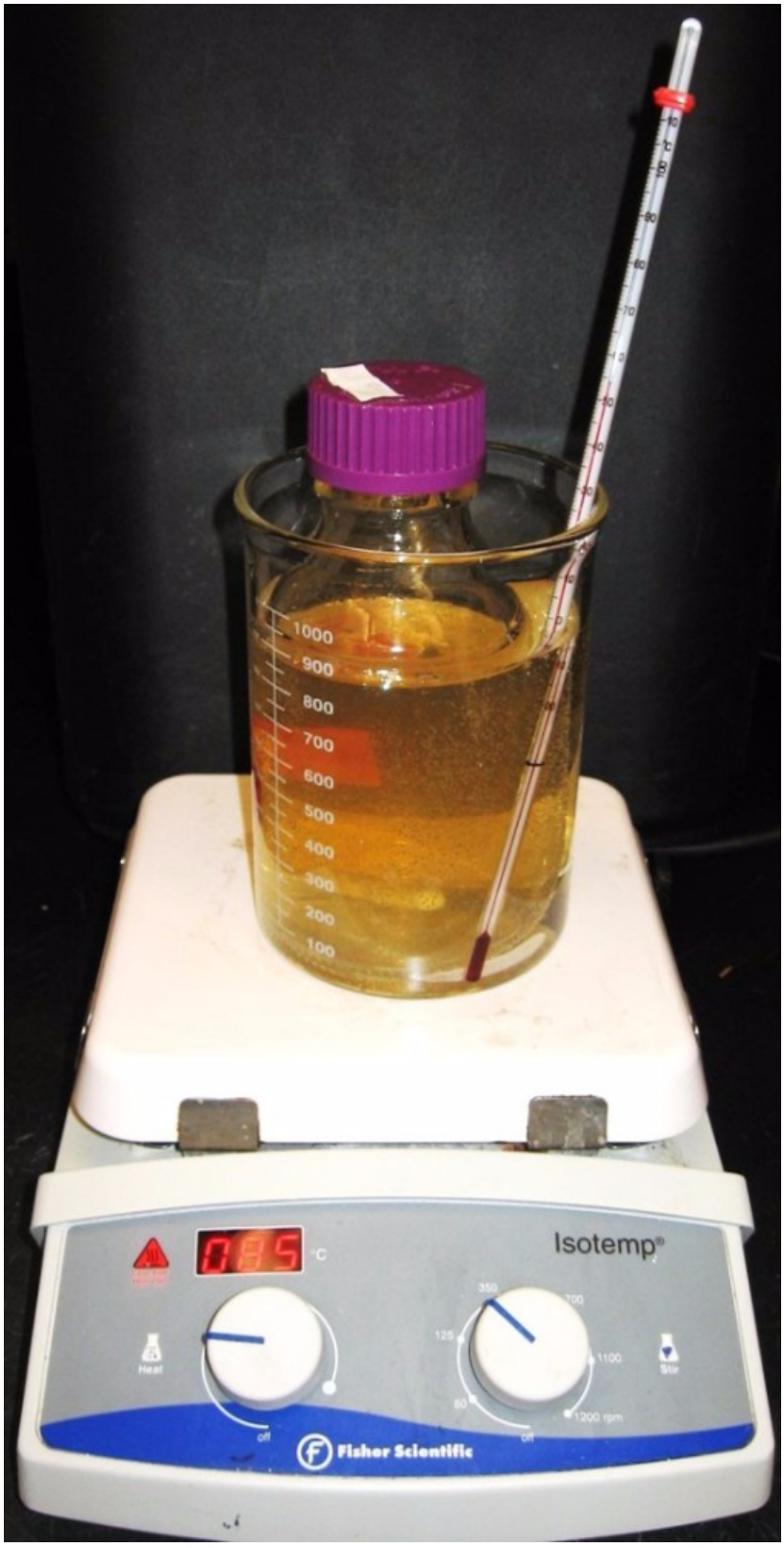


Figure 1. Top agar bottle equilibrated in beaker-waterbath.

NOTE

Note: This will require setting the heat block to a temperature greater than the target temperature, for example up to 85°C, but this is dependent on specific heat block models and must be determined by the user.

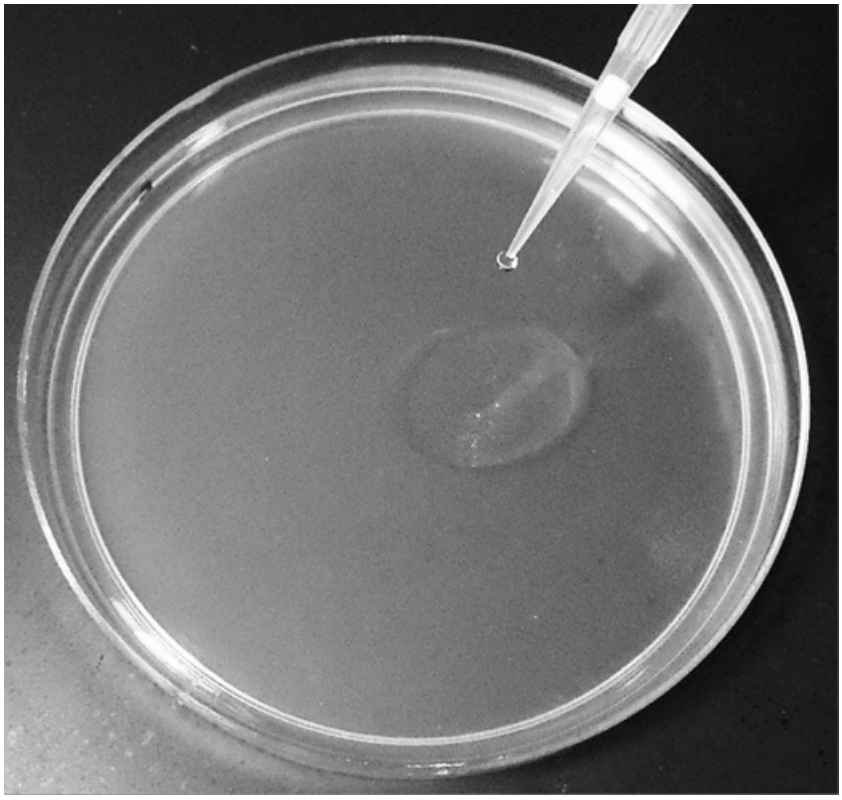
II. Procedure for Tube-free Agar Overlays

4

Add host: Pipette 100 ul of overnight host culture directly onto the bottom agar .

5

Add virus: Pipette 10-100 ul of virus-containing solution directly into the host droplet on the bottom agar.

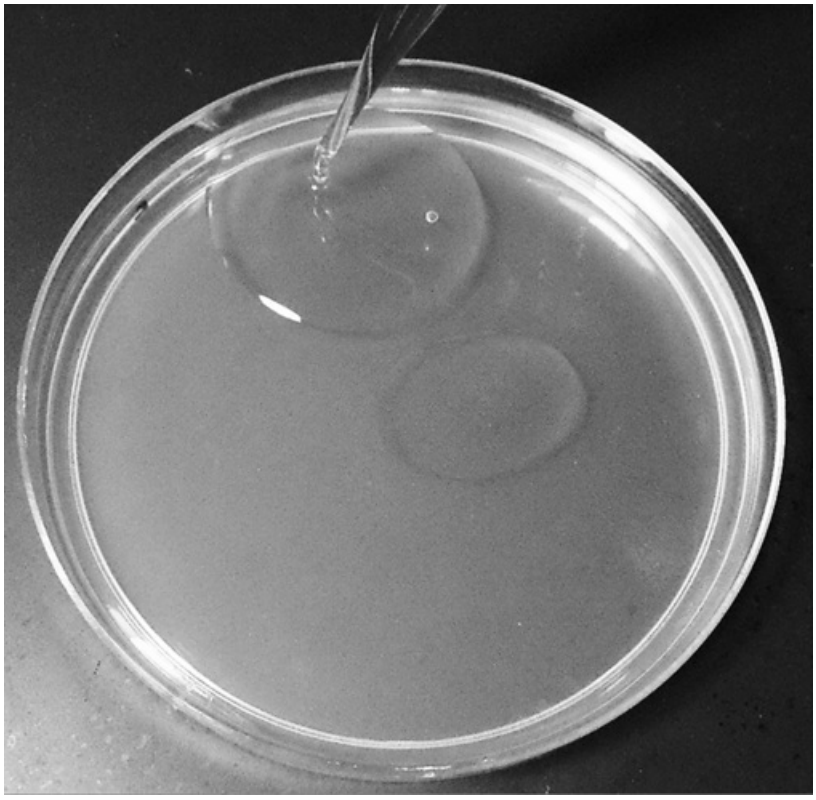


6

Add molten top agar:

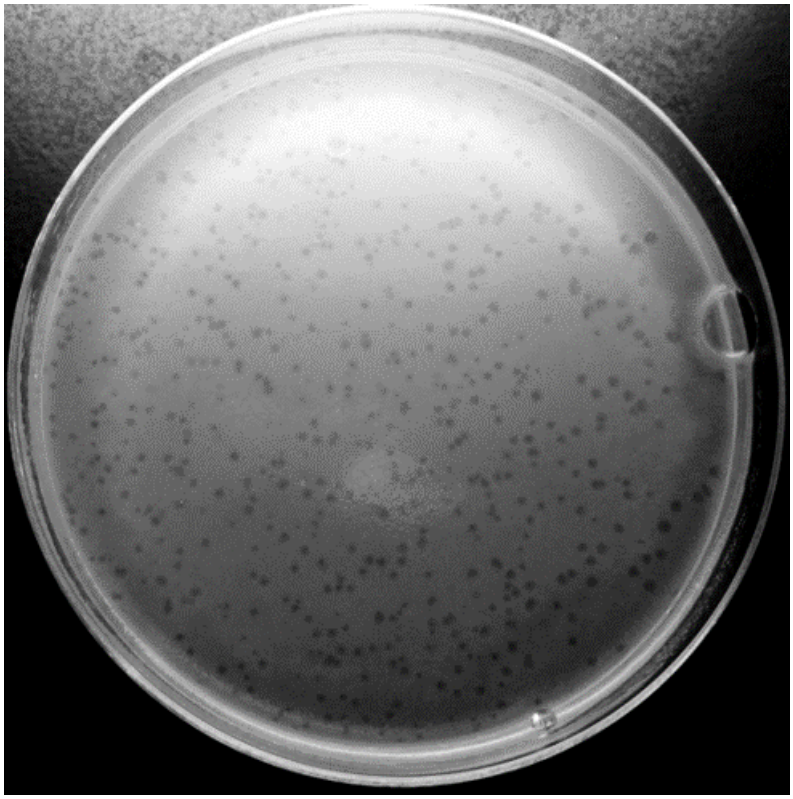
Remove the lid of the top agar bottle briefly to pipette out 2 ml of molten top agar using either a 5 ml pipette or serological pipette, and sterile technique.

Pipette the 2 ml of molten top agar from the bottle directly onto the bottom agar next to the droplet of hosts and viruses.



7 **Make overlay:** Swirl the plate vigorously but briefly to mix the bacteria and virus and molten top agar and to spread it across the plate

8 **Set and incubate:** Leave the plate on the bench, top agar side up, for at least 20 minutes to allow the agar to completely solidify, then place in desired incubation conditions and monitor for plaque formation.



NOTE

Note: Failure to achieve satisfactory lawns is often due to either: 1) insufficiently melted top agar, which gives rise to matte lawns instead of glossy smooth lawns, and 2) taking too long to swirl the agar thus allowing it to cool down and set before the mixing is completed.

NOTE

Note: As in all plating assays, it is recommended that control plates be included to ensure that: 1) the agar overlay is contaminant free at the start and finish of the experiment (include only top agar in these controls), 2) the bacterial stock is virus-free (include only bacteria and top agar in thee controls), and 3) the virus stock is not contaminated with cells (include only virus stock and top agar in these controls).