# Bacteriophage Solid Propagation in *Pseudomonas aeruginosa*

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**Method Article**

**Keywords:** bacteriophage, phage, propagation, amplification, solid production, plate production, soft agar, agar, Pseudomonas aeruginosa, podovirus

**Posted Date:** January 19th, 2023

**DOI:** https://doi.org/10.21203/rs.3.pex-1953/v1



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# Abstract

This protocol provides a solid production method for bacteriophages (phages) based on co-incubation of host bacteria and phages in soft agar on an agar media plate. The phages are then collected by an overlay of SM-buffer and through mechanical dispersion of the soft agar in the SM-buffer. This protocol has the same cost-effectiveness as liquid propagation approaches, but requires more working time (40 min), due to the initial preparation of the agar media plates and the subsequent way of collecting the produced phages. As some phages are difficult to produce to high titers in liquid cultures, this protocol provides an alternate approach.


# Introduction

The propagation of phages, to increase their concentration and quantity, constitutes a central element when working with phages required for many downstream analysis and applications. When propagating phages, there are two main approaches: relying on a liquid medium or a solid agar medium (this protocol) as the culture environment. Before deciding on either approach, both should be tested out for the particular phage-host bacteria combination. The protocol that yields the best results should then be further adapted to the specificities of the phage-host bacteria interaction (see Troubleshooting).

# Reagents

1. Agar (Sigma-Aldrich, St. Louis, USA; cat. no.: 05040-250G)
2. Double-distilled water (dd$H_2O$)
3. Hydrochloric acid (HCl 37%; Carl Roth GmbH, Karlsruhe, Germany; cat. no.: 7476.2)
4. Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$; Sigma-Aldrich, St. Louis, USA; cat. no.: M2773)
5. TRIS ($C_4H_{11}NO_3$; Carl Roth GmbH, Karlsruhe, Germany; cat. no.: 2449.1)
6. Tryptic soy broth (TSB; USBiological, Salem, USA; cat. no.: T8727-10)
7. Sodium chloride (NaCl; Molekula GmbH, Munich, Germany; cat. no.: 41272436)
8. Tryptic soy agar (TSA; 3% w/v tryptic soy broth powder + 1.5 % w/v agar powder)
9. Tryptic soy soft agar (soft agar; 3% w/v tryptic soy broth powder + 0.6 % w/v agar powder)
10. Saline magnesium buffer (SM-buffer): Prepare 1 M Tris-HCl buffer (pH 7.5) in a 100 ml bottle by adding 6.06 g of TRIS to 50 mL dd$H_2O$ and adjusting the pH to 7.5 with hydrochloric acid. Then, add in

an autoclaved 1 l bottle 5.8 g of sodium chloride, 2 g of magnesium sulphate and 50 ml of the prepared 1 M Tris-HCl (pH 7.5), and adjust to 1 l with dd$H_2O$.

# Equipment

1. Centrifuge (e.g., Eppendorf 5430 R; Eppendorf, Hamburg, Germany; cat. no.: 5428000205)
2. Centrifuge tube (50 ml and 15 ml; Falcon® Corning Inc., Corning, USA; cat. no.: 352070 and 352096)
3. Incubator (e.g., Incubator IN110; Memmert GmbH, Schwabach, Germany; cat. no.: IN110)
4. Inoculation loop (VWR International, Pennsylvania, USA; cat. no.: 612-9358)
5. L-shaped spreader (VWR International, Pennsylvania, USA; cat. no.: 612-1561)
6. Microcentrifuge tube (1.5 ml Eppendorf Safe-Lock Tube; Eppendorf AG, Hamburg, Germany; cat. no.: 0030120086)
7. Petri dish (VWR International, Pennsylvania, USA; cat. no.: 391-0467)
8. Pipette controller and serological pipettes (5 ml and 10 ml)
9. Pipettes and pipette tips (200 µl and 1000 µl)
10. Refrigerator
11. Shaker thermoblock (e.g., Thermomixer Comfort 5355 Block 24 x 2.0 ml; Eppendorf AG, Hamburg, Germany; cat. no.: 5355 000.011)
12. 0.45 µm filter (VWR International, Pennsylvania, USA; cat. no.: 514-0063)
13. 0.22 µm filter (Merck Millipore, Massachusetts, USA; cat. no.: SLGSM33SS)
14. 20 ml Syringe (BD; New Jersey, USA; cat. no.: 300629)

# Procedure

1. Prepare a bacterial culture by pouring an appropriate volume of TSB broth (e.g., 5-10 ml of 3% w/v) into a centrifuge tube and adding the frozen aliquot of bacteria or, using an inoculation loop, selecting an isolated colony from the media plate and shaking the loop in the TSB broth media until the fragment has come loose.

2. Seal the centrifuge tube with the lid and incubate it overnight (approximately 12-24 h) at 37 °C and 150 rpm in an orbital shaker.

3. Prepare a phage-host bacteria culture by adding 800 µl of TSB, 100 µl of overnight grown bacterial culture and 100 µl of phage stock solution (at a final titter: $10^5$ PFU/ml) to a 1.5 ml microcentrifuge tube.

4. Mix the culture by pipetting up and down 10 times (do not vortex the solution).

5. Place the culture in a shaker thermoblock and incubate for 1 h at 37 °C and 300 rpm.

6. Add in a 15 ml centrifuge tube 200 µl of the culture and 2.5 ml of molten soft agar (50 °C) and immediately pour the mixture into a TSA plate. Prepare four plates per culture.

7. Wait until the soft agar solidifies completely and place the four plates in an incubator overnight at 37 °C.

8. Overlay the plates with 3 ml of SM-buffer and keep them with the lid closed at room temperature for 30 min.

9. Transfer the SM-buffer into a 50 ml centrifuge tube.

10. Using a L-shape spreader scratch the soft agar from each plate and transfer it into the centrifuge tube.

11. Thoroughly mix the resulting SM-buffer/soft-agar mixture with an inoculation loop.

12. Centrifuge the mixture for 10 min at 4 °C and 4000 g.

13. Filter the supernatant with a 0.45 µm syringe filter and consecutively with a 0.22 µm syringe filter into a sterile centrifuge tube.

14. Store the solution in a refrigerator at 4 °C until further use.

# Troubleshooting

Arising from the immense diversity of phages, there is not one optimal production method for all phage-host bacteria combinations. Therefore, it is advisable to test different production methods and adjusting the parameters (see below) to the specificities of the phage-host bacteria interaction.

Step: 2, 5 & 7

Problem: Temperature

Possible reason: Different bacteria have differing optimal growth temperatures

Solution: Increase/decrease the temperature

Step: 3

Problem: Phage concentration

Possible reason: Different phages work best at differing MOIs(multiplicity of infection)

Solution: Increase/decrease the starting concentration of the phages

Step: 5

Problem: Agitation

Possible reason: Hindering phage adsorption to bacteria

Solution: Decrease the rpm

Step: 5

Problem: Incubation time

Possible reason: Some phage-host bacteria combinations might need different co-incubation times

Solution: Increase/decrease the duration of co-incubation from 10 h up to 48 h

Step: 6

Problem: Soft agar concentration

Possible reason: Some phages may have poor diffusion in agar

Solution: Decrease the soft agar concentration (0.3 % - 0.4 %) or replace agar by agarose, in even lower concentration (0.2 %)

Further adaptations may include the supplementing of the medium with divalent cations (e.g., $CaCl_2$, $MgSO_4$) and the use of chloroform or -80 °C to liberate phages from bacteria remaining in the supernatant after centrifugation (step 12). The incubation time (step 7) can also be coupled with culture clearance (soft agar layer with confluent lysis), which might occur after several hours, as phages propagate and lyse bacterial host cells.

## Time Taken

Besides the overnight incubation of the plates the time needed amounts to approx. 2:20 h, including the 1:40 h of waiting time.

## Anticipated Results

With this protocol, phage concentrations of $10^9$ to $10^{10}$ PFU/ml are expected to be reached for several *P. aeruginosa* bacteriophages of podovirus morphology.

## References


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## Acknowledgements


We thank the countless researchers on ResearchGate who answer questions, spark new ideas and highlight paths which might yield higher phage titers and improve phage propagation protocols.