Plaque plating

There are two methods that have been routinely employed at Sapphire to make plaque plates. One method simply plates a mixture of cells and infection directly onto the agar plate. The second method uses a soft agar overlay for the plating where a mixture of cells and infection source is added to molten soft agar (media with a low % of agar) and then poured onto a plate, forming a thin layer on top of the agar media below. The media used for the overlay is typically the standard laboratory media for the host algae in question.

Direct vs. agar overlay plating

The advantage of the direct plating method is that it is easier since it requires less steps and equipment and as long as you have plates in the deli it can be done at any time. The advantage of the agar overlay method is that it yields more consistent plaque size and morphology as well as generating a more homogenous lawn of the host organism. These observations are from literature and personal communications with phage biologists and algal (chlorella) virologists as well as some experience. The advantages may not hold true for other host/pest combinations and equally there may be other advantages for either technique in these novel situations. Some pests may be so temperature sensitive that the soft agar overlay method could be detrimental. When discussing plaque plates with Dave Dunigan for example he said that the chlorella viruses would be inhibited at 55°C and so it was very important to have the water bath no higher than 50°C. The method should work fine as low as 45°C but at this lower temperature you may need to work more quickly or with fewer samples at a time since the media will solidify faster.

Plates

Agar plates may need to be poured slightly thinner than normal for the soft agar overlay technique. This will depend somewhat on the plates and how thick you normally pour the plates. When using the Fisher plates (cat#FB0875712) pour approximately 25ml for the standard/underlay plate. This should allow sufficient room to pour the 4-5ml of soft agar/sample on top. Plates from VWR are not as deep or wide so care should be taken when pouring plates to leave enough room for the overlay layer.

Soft agar is typically made with 0.4-0.6% w/v agar and at Sapphire we have used 0.5%w/v. Soft agar can be made at the time of the procedure and left in the 55C incubator until ready for use as with other solid media recipes. It can also be made ahead of time in small bottles (e.g. 100-200ml) and then stored as solid media. When needed it can be melted in the microwave (with caution!) or a boiling water bath before use. Soft agar can be re-melted for use multiple time.

When we worked with Dave Dunigan on the Chlorella viruses he suggested that plates should be fresh (up to 3 days old) for good viral plaque formation and that more erratic results were seen with older plates. It is unclear where this information comes from or if it translates to other organisms.

Optimization of conditions for plaque formation

For any system with a host/pest interaction there are many opportunities for optimization. It is important that conditions for growth and infection are balanced so that one is not overwhelming the other. In the extreme cases this could mean no plaques at all and only a lawn of the host survives, or the opposite situation where the host is completely decimated very early on. Adjusting the amount of host cells plated per plate will also have an effect in a similar way.

Some work was done in San Diego trying different light and CO2 levels (+/-) with SE00004 and various fungal pests. I do not have the data for these studies but what I saw suggested that slower growth of the algae in this case (lower light, no CO2) produced better plaques. However, without knowing all the details this information should be taken lightly and it is likely that the ideal conditions will be different for different pests/hosts.

Certain media additions may be required for infection. For example, the level of Ca or Mg is important for some bacteriophage infections. If infections do not occur in standard lab media then trying a media that more closely resembles the environment where the pest was found may improve success.

Safety

Wear gloves and work in the flow hood with unknown organisms and/or environmental samples. Avoid steps that generate aerosols in the lab such as vortexing without lids. Regularly clean pipettes to prevent cross contamination.

Materials required

1. Agar plates of appropriate media
2. Soft agar of appropriate media (if using agar overlay method)
3. Tranny balls or hockey stick (is using direct plating method)
4. Liquid media for dilution of infection
5. Vessel for dilution (e.g. 96-well plate or microfuge tubes)
6. "preview" tubes, capped and sterile (plastic test tubes large enough to hold 10+ml)
7. Pipettes and tips
8. Pipette filler and serological pipettes
9. 45-50°C water bath (if using agar overlay method)
10. Host strain in log phase growth
11. Pen or label maker

Procedure for agar overlay

1. Gather supplies, clean and wipe work area with 70% Ethanol. Refer to Laminar flow hood use SOP LC-01-001-018 for proper hood use.
2. Turn on water bath and allow to heat up to 50°C.
3. Melt the soft agar and hold in the 50°C water bath.
4. Dispense 2.5 ml of soft agar to sterile "preview tubes" using a serological pipette and filler and place in a test tube rack in the water bath at 50°C. Do this for the number of plates that will be poured plus a few spares.
5. Concentrate the host strain to 4.0 X 10^8 cells/ml by centrifugation and re-suspension with fresh media. Concentrate enough so that 0.3 ml can be used for each plate plus some extra for backup.
6. Dilute the pest sample in fresh media by 1/10 serial dilutions in a volume sufficient to allow 0.1ml of each dilution to be plated on the number of plates you intend to use. The volume needed will determine the best vessel for this but sterile 96-well plates work well and allow the use of multichannel pipettes which can be useful if using multiple pest sources. How far to dilute the pest will depend on the host/pest system. For an unknown sample/pest dilute source to at least 10^{-6}.
7. Label the plates with at least date, initials, host strain, pest sample, dilution. Use pen to write on the edge of the base of the plate or attach a label towards the edge of the plate.
8. Remove 13 x 100 mm tubes of soft agar from the water bath to a test tube rack as needed. Start by doing only 1-2 at a time until you are familiar with how long it takes to complete the task before the media solidifies. Once you are comfortable with the procedure the number of tubes handled at one time can be increased.
9. To each tube, add by pipette 0.3 ml of the concentrated host and 0.1 ml of the diluted pest.
10. Mix briefly (by rolling the tubes between the palms of the hands) and pour the contents of the tube onto the corresponding plate.
11. Tilt the plate gently until the entire surface of the plate is covered with soft agar (this needs to be done quickly, as the soft agar will start to solidify as soon as it has been poured onto the plate).

12. Allow the agar to solidify (a few minutes).
13. Invert the plates with the lid down (so moisture condensation stays in the lid and off the agar surface)
14. Turn off the water bath, tidy up and clean and wipe down work area with 70% Ethanol.
15. Incubate the plates at appropriate conditions. If ideal conditions for the target pest have not yet been established, make duplicates plates and place in multiple conditions to compare results.
16. Expect plaques to appear in 5-15 days.

Procedure for direct plating without agar overlay

1. Gather supplies, clean and wipe work area with 70% Ethanol. Refer to Laminar flow hood use SOP LC-01-001-018 for proper hood use.
2. Concentrate the host strain to 8.0 X 10^8 cells/ml by centrifugation and re-suspension with fresh media. Concentrate enough so that 0.15 ml can be used for each plate plus some extra for backup.
3. Dilute the pest sample in fresh media by 1/10 serial dilutions in a volume sufficient to allow 0.05ml of each dilution to be plated on the number of plates you intend to use. The volume needed will determine the best vessel for this but sterile 96-well plates work well and allow the use of multichannel pipettes which can be useful if using multiple pest sources. How far to dilute the pest will depend on the host/pest system. For an unknown sample/pest dilute source to at least 10^{-6}.
4. Label the plates with at least date, initials, host strain, pest sample, dilution. Use pen to write on the edge of the base of the plate or attach a label towards the edge of the plate.
5. If using plating balls for plating add them to each plate.
6. Collect and label sample vessels. Any suitable sterile vessel can be used for this step. If multiple plates of the same dilution are to be plated then a master mix can be made.
7. To each sample vessel, add by pipette 150ul of the concentrated host and 50ul of the diluted pest.
8. Mix briefly by pipetting up and down and pipette 200ul of the mixture onto the corresponding plate.
9. Spread sample over plate with desired technique.
10. Allow sample to dry/adsorb with the plate facing up.
11. Once dry, invert the plates with the lid down (so moisture condensation stays in the lid and off the agar surface)
12. Wrap plates if desired. If using parafilm, poke 1-2 holes to allow air flow and to reduce moisture build up.
13. Tidy up and clean and wipe down work area with 70% Ethanol.
14. Incubate the plates at appropriate conditions. If ideal conditions for the target pest have not yet been established, make duplicates plates and place in multiple conditions to compare results.
15. Expect plaques to appear in 5-15 days.