REPLICATING COLONIES ONTO NITROCELLULOSE FILTERS

Procedure I

This procedure (Hanahan and Meselson 1980, 1983) is used when you know ahead of time that you will need to screen large numbers of colonies. In this case, bacteria are plated directly from a transformation mixture onto detergent-free nitrocellulose filters; replica filters are prepared by filter-to-filter contact.

1. Number the dry nitrocellulose filters with a soft pencil or a ball-point pen and sterilize them as described on page 304. Prepare enough filters for a master and two replicas.

2. Using sterile, blunt-ended forceps, lay a sterile filter, numbered side down, on a day-old agar plate containing the appropriate antibiotics. Peel off the filter, invert it, and replace it on the plate, numbered side up.

3. Apply the bacteria in a small volume of liquid (< 0.8 ml, containing up to 20,000 bacteria for a 137-mm filter; < 0.4 ml, containing up to 10,000 bacteria for an 82-mm filter). Spread the liquid over the surface of the filter with a sterile, bent glass rod. Leave a border 2–3 mm wide at the edge of the filter free of bacteria. Let the plates stand at room temperature until all of the liquid has been absorbed.

4. Invert the plates and incubate at 37°C until very small colonies (0.1-mm diameter) appear (about 8–10 hours).

5. Prepare in advance a stack of Whatman 3MM papers sterilized by autoclaving (one for each filter plus a few spares)

6. Wet a numbered, sterile nitrocellulose filter (Millipore HAWP) by touching it to the surface of an agar plate containing the appropriate antibiotic. Leave the filter in contact with the surface of the agar, numbered side up. Try to arrange that the numbers on the replica filters correspond to those on the master filters.

7. Using sterile, blunt-ended forceps (e.g., Millipore forceps), gently remove the master filter from the first plate and place it on the stack of 3MM paper, colony side up.

8. Carefully place the second, wetted filter (numbered side down) on top of the master, being careful not to move filters once contact has been made. Press the filters together using sterile velvet stretched over a replica-plating tool (see Fig. 9.7, page 306).

9. Use an 18-gauge needle to make a characteristic pattern of keying holes in the filters while they are sandwiched together. Gently peel the filters apart and return the second filter to its plate, colony side up.

10. Prepare a second replica from the master in an identical manner. Key the replica to the existing holes in the master filter. Return both filters to their respective plates. If the master filter is to be used to make more than two replicas, it should be incubated for a few hours to allow the colonies to regenerate. Generally, making two to three replicas presents no problem. However, should more replicas be required, it is essential that the bacterial colonies be extremely small at the time of replication in order to avoid smearing.

11. Incubate the plates (master and replicas) at 37°C until colonies 1–2 mm in diameter have appeared. Colonies on the master plate reach the desired size more rapidly (6–8 hours).

    At this stage, while the bacteria are still growing rapidly, the replica filters may be transferred to agar plates containing chloramphenicol (170–250 μg/ml) and incubated for a further 12 hours at 37°C. As discussed on page 313, this amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low.

12. Seal the master plates with parafilm and store at 4°C in an inverted position until the results of the hybridization reaction are available.

13. Lyse the bacteria and bind the liberated DNA to the replica filters using one of the two procedures described on pages 314–315.

Procedure II

This method is used to transfer many bacterial colonies simultaneously from the surface of agar plates to nitrocellulose filters. The method works with bacterial colonies of any size, but small colonies (0.1–0.2 mm) give the best results; they produce sharper hybridization signals and smear less than larger colonies. As many as 10^4 colonies per 150-mm plate can be screened using this technique.

1. After the bacterial colonies have grown to a diameter of 0.1–0.2 mm, remove the plate from the incubator and store it for 1–2 hours at 4°C in an inverted position.

2. Label a dry nitrocellulose filter (Millipore HAWP) with a soft pencil or ball-point pen and place it, numbered side down, on the surface of the agar medium in contact with the bacterial colonies until it is completely wet. Mark the filter and underlying agar in three or more asymmetric locations by stabbing through it with an 18-gauge needle attached to a syringe containing waterproof black drawing ink (e.g., Higgins India Ink).

   Although sterile filters are to be preferred, nonsterile filters can also be used as long as the master plate is not to be used again to make replicas.

3. Using blunt-ended forceps, peel off the filter.

4. At this stage several options are available:

   a. The bacteria adhering to the filter can be lysed immediately and the liberated DNA bound to the filter using one of the two procedures described on pages 314–315.

   b. The filter can be placed, colony side up, on the surface of a fresh agar plate containing the appropriate antibiotics. After incubation for a few hours, the large bacterial colonies 2–3 mm are lysed. This method is necessary only when transfer of the colonies to the filter is poor or uneven. This is not usually the case.

   c. The filter can be placed on a fresh agar plate containing chloramphenicol (10 μg/ml). As discussed earlier, this amplification is used only when the copy number of the recombinant plasmid is expected to be low.

   d. The filter can be used to prepare a second replica. The filter is placed, colony side up, on the surface of a fresh agar plate containing the appropriate antibiotics. A second, dry nitrocellulose filter is then laid carefully on top of the first and keyed to it. The filter sandwich is incubated for several hours at 37°C and the plasmids are amplified, if

desired, by further incubation on an agar plate containing chloramphenicol. The filters are kept as a sandwich during the subsequent lysis and neutralization steps but are peeled apart before the final wash (Ish-Horowicz and Burke 1981).

5. Incubate the master plate for 10–12 hours at 37°C until the colonies have regenerated. Seal the plate with parafilm and store at 4°C in an inverted position.