Video Article

Large-Scale Screens of Metagenomic Libraries

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Abstract

Metagenomic libraries archive large fragments of contiguous genomic sequences from microorganisms without requiring prior cultivation. Generating a streamlined procedure for creating and screening metagenomic libraries is therefore useful for efficient high-throughput investigations into the genetic and metabolic properties of uncultured microbial assemblages. Here, key protocols are presented on video, which we propose is the most useful format for accurately describing a long process that alternately depends on robotic instrumentation and (human) manual interventions. First, we employed robotics to spot library clones onto high-density macroarray membranes, each of which can contain duplicate colonies from twenty-four 384-well library plates. Automation is essential for this procedure not only for accuracy and speed, but also due to the miniaturization of scale required to fit the large number of library clones into highly dense spatial arrangements. Once generated, we next demonstrated how the macroarray membranes can be screened for genes of interest using modified versions of standard protocols for probe labeling, membrane hybridization, and signal detection. We complemented the visual demonstration of these procedures with detailed written descriptions of the steps involved and the materials required, all of which are available online alongside the video.

Protocol

1. Hybridization procedure

One hybridization tube will take 1 or 2 membranes (size 22 by 22 cm). Use 50 ml of hybridization mixture and 0.5 mg of DNA probe (10 ng/ml final concentration) per hybridization tube. For best results, use gel-purified DNA as probe. If scaling up probe preparation, increase number of tubes rather than increasing the amount of reagents per tube.

Probe preparation (0.5 mg DNA):

1. Dilute 15 µl of cross-linker solution with 60 µl of water.
2. Dilute DNA to 10 ng/µl with water to 50 µl total volume in a screw-capped microtube.
3. Denature DNA for 5 min in boiling water.
4. Rapidly transfer to ice and let cool for 5 min. Spin briefly.
5. Add 50 µl of reaction buffer and mix gently.
6. Add 10 µl of labeling reagent and mix gently.
7. Add 50 µl of the diluted cross-linker solution from step 1 and mix gently. Vortex gently and spin briefly.
8. Incubate at 37°C for 30 min.
9. Keep on ice for up to 2 h or add an equal volume of 100% glycerol and store at -20°C.
   Note: Use the water supplied with the kit. Keep all reagents on ice unless otherwise stated.

Membrane preparation and hybridization

1. Preheat 50 ml of hybridization buffer and hybridization tube to 60°C.
2. Add the hybridization buffer to the hybridization tube. Roll up membrane(s) and add to hybridization tube. (If adding 2 membranes per tube, first roll up one membrane completely, then roll up the other membrane on top in the same direction.)
3. Incubate hybridization tube at 60°C for at least ½ h. (Make sure membrane(s) get soaked thoroughly.)
4. Transfer about 1 ml of hybridization buffer with a long pipette from the hybridization tube to the tube containing the labeled probe. Vortex gently and spin briefly. Transfer mixture back to hybridization tube.
5. Incubate at 60°C overnight.
   Note: Use forceps and gloves when handling membranes. Mix content of hybridization tubes gently because too much agitation will cause permanent formation of undesirable foam. Membranes should be added in wet condition; soak in hybridization buffer or water if necessary and drain off excess liquid before adding.

2. Detection procedure

Washing membranes

1. Preheat primary washing buffer to 60°C. (Use microwave oven and/or heating plate.)
2. If there are 2 membranes in one hybridization tube, transfer one membrane to a clean, preheated, empty hybridization tube.
3. Briefly rinse hybridization tube with about 50 ml of primary wash buffer.
4. Add about 100 ml of primary wash buffer to hybridization tube and incubate at 60°C for 10 min.
5. Repeat step 3 and 4.
6. Transfer membrane to washing container and incubate with at least 250 ml of secondary wash buffer at room temperature for 5 min.
7. Repeat step 6.
   Note: Membranes may be kept in the secondary wash buffer at room temperature for up to ½ h before signal generation.

Signal generation and detection (procedure for one membrane)

1. Tape two pieces of SaranWrap flat onto the tabletop (one for step 2 and one for step 4).
2. Carefully drain off excess buffer from the membrane by touching the corners of the membrane against the side of the container and transfer the membrane onto the SaranWrap (sample side up).
3. Pour 6-7 ml of ECF reagent over membrane and fold Saran wrap over membrane. Carefully distribute ECF reagent over membrane by gently moving a Kimwipe over the SaranWrap. Incubate for 5 min.
4. Drain off excess ECF reagent from the membrane and transfer it onto new SaranWrap, fold wrap over membrane, and seal the edges with tape. Cover the membrane with aluminum foil. Incubate for 1-2 hours.
5. Prepare the scanner glass plate by distributing 1-2 ml of ECF reagent over the glass plate. Then transfer the membrane onto the glass plate (sample side down) and distribute another 1-2 ml of ECF reagent over the membrane. Overlay with SaranWrap and carefully push all air bubbles away from the membrane. (You may place a stiff plate on top of the SaranWrap to keep membrane pressed against the glass plate.)
6. Use the Fuji FLA-5100 fluorescence scanner ("1 laser 1 image" mode) with the following settings: excitation 473 nm, detection filter LPB (510 nm), resolution 50 µm, 16-bit signal graduation, CH1 400 V. (Scan takes about 15-20 min per membrane of size 22 by 22 cm.)
   Optional:
7. Let sit for 2-4 hours (or longer).
8. Repeat scan as in step 6.

3. Stripping and storing procedure (NUF version)

1. Transfer membrane to 100% ethanol and incubate at room temperature for 10 min with gentle agitation.
2. Replace with fresh 100% ethanol and incubate overnight at room temperature. (Membrane may remain in ethanol for several days.)
3. Replace ethanol with distilled water and incubate at room temperature for 10 min.
4. Add 100 ml water and 5 ml of 10% SDS (final 0.5% SDS) to a hybridization tube and mix gently. Add membrane.
5. Incubate at 80°C for 1 h.
6. Transfer membrane to a clean, empty container.
7. Add 500 ml water and 5 ml of 10% SDS (final 0.1% SDS) a beaker and heat to a vigorous boil in the microwave oven.
8. Pour the near-boiling 0.1% SDS solution over the membrane and gently agitate for several minutes. Allow to cool to room temperature.
9. Rinse in at least 250 ml of sterile 100 mM Tris pH 8 for at least 10 min.
10. Wrap membrane in SaranWrap and carefully close edges with tapes. Store at 4°C. (Membranes must never dry out.)

Discussion

The stripping procedure has not been optimized. However, the stripping procedure given in the manufacturer's instruction (2 washes each, 10 min in 100% ethanol, 1 wash of 1 h at 60°C in 0.5% SDS) is insufficient. At least overnight incubation in 100% ethanol is necessary to dissolve the ECF reaction product; several days incubation in 100% ethanol appears to have no adverse effects on the ability to reprobe the membrane. The manufacturer's SDS treatment also appears to be insufficient. NUF has tried 1 h at 80°C with 0.5% SDS followed by one treatment with near-boiling 0.1% SDS with success. Tracy has shown that one time pouring-over with near-boiling 0.1% SDS is sufficient.

Helpful hints:
The hybridization buffer, hybridization tube, and primary wash buffer must be preheated to 60°C prior to use, and must be kept at that temperature throughout the experiment. For example, keep the buffers on a heating plate with an immersed thermometer; on a Corning heating plate, a setting of about 3 results in 60°C.

References

