

Supplemental Protocol for Colony Blot Hybridization

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I. Determine Serial Dilution for Dilution Plating TAC pools

1. Thaw TAC library pools of interest on ice for 2 hours. Gently shake tubes after thaw is complete.
2. Take 20 μ l of TAC pool of interest, place in 600 μ l tube and freeze at -80C. Take 2 μ l of TAC pool of interest and inoculate into 198 μ l of LB + Kanamycin broth (Km concentration is 25mg/L). This is 10^{-2} dilution. Also do a 10^{-3} (inoculate 20 μ l from 10^{-2} dilution into 180 μ l LB + Km broth), a 10^{-4} , and a 10^{-5} serial dilution.
3. Mix each serial dilution with vortex and pipette 100 μ l onto 100 x 15mm LB + Km agar plate (2-3 days old) using aseptic technique. Put all agar plates at 37C for 2-3 hours prior to plating bacterial suspension. Place remainder of original bacterial suspension in cold room.
4. Incubate inverted plates for 16 hours at 37C.
5. Chose plates with distinct colonies, divide plates of interest into fourths and count 1/4 of the plate. Extrapolate to determine #colonies/plate and chose correct serial dilution for colony blot hybridization.

II. Dilution Plating TAC pools and Colony Lift

1. Use the 20 μ l of the frozen TAC pool of interest to create the serial dilution of interest. Plate 250 μ l of serially diluted bacterial suspension directly onto the surface of 150 x 15mm LB + Km agar plates that are 2-3 days old. A total of 5,000 clones are needed from each pool to accurately screen. Determine the number of plates needed from the original serial dilution exercise in I. Leave the plates with their lids ajar in the hood until the agar surface is dry. Close the lids and incubate inverted plates at 37C for 16 hours. Chill the plates for 30-60 minutes at 4C.
2. Number all filters on one side and on the other side, mark each filter in 3 asymmetric locations with graphite pencil. Place numbered, sterilized Nylon + filters number side up, on the surface of the LB + Km agar medium in contact with the bacterial colonies, until it is completely wet. Lift the plate and mark the bottom of the plate with a sharpie where the filter is marked 3 times.
3. Using blunt-end forceps, peel off the filter and go to III.
4. Incubate the master plate for 4-6 hours at 37C until the colonies have regenerated and then seal the plate with Parafilm and store at 4C in an inverted position.

III. Lysis of Colonies and Binding of DNA to Nylon + Filters

*Try to avoid getting any of the solutions onto the upper surface of the filter and trapping air bubbles under the filter.

*Can transfer filter to dry paper towel between steps to remove excess liquid.

1. Cut 4 pieces of Whatman 3MM paper to fit neatly into the bottoms of 4 glass trays. Saturate each piece of 3MM paper in one of the 4 following solutions:
 - *10% SDS (optional)
 - *Denaturing Solution (0.5N NaOH, 1.5M NaCl)
 - *Neutralizing Solution (1.5M NaCl, 0.5M Tris Cl pH7.4)
 - *2 x SSCPour off any excess liquid
2. Take the filters and place them colony side up on SDS 3MM paper. This step limits the diffusion (spreading) of the plasmid DNA which will give a sharper hybridization signal. Can also do these steps with one filter at a time.
3. After the filter has been exposed to the SDS 3MM paper for 3 minutes, transfer it to the Denaturing Solution 3MM paper and expose for 5 minutes.
4. Transfer the filter to the Neutralizing Solution 3MM paper and expose for 5 minutes.
5. Transfer the filters to the 2 x SSC 3MM paper and expose for 5 minutes.
6. Lay filters, colony side up, on a sheet of dry 3MM paper. Allow to dry at room temperature for at least 30 minutes.
7. Bind DNA to the filters using a UV Stratalinker (30 seconds). After the DNA has been bound to the filter, wrap the filter in Saran Wrap and place at 4°C until ready to hybridize.

IV. Probe Labeling with DECAprime II DNA Labeling Kit

1. Perform PCR reaction with primers of interest and LA407 template DNA. Gel purify PCR product with QUIAGEN kit and resuspend DNA in ddH₂O. Dilute DNA to be labeled in ddH₂O to a concentration of 1-2.5ng/μl.
2. A standard DECAprime II reaction will accommodate 6-100ng of DNA to a volume of up to 11.5μl. The higher the concentration of the template DNA, the more probe synthesized, but the less the specific activity of the probe. Recommend 25ng as the ideal amount of template DNA
3. Add 10μl of DNA to microfuge tube with 2.5μl of 10x decamer solution. Cap tube and place in heat block/H₂O bath at 95-100°C for 3-5 minutes to denature DNA.
4. Snap-freeze in liquid nitrogen or dry-ice ethanol to prevent self-annealing of template DNA. Thaw, microfuge and place on ice.
5. Add the following to the tube on ice:

12.5μl	Denatured DNA/Decamer Mix from step 4
5.0μl	5x buffer (-dCTP)
5.0μl	[α- ³² P] dCTP (3000 Ci/mmol)
1.5μl	of nuclease-free ddH ₂ O (autoclaved) to make final volume of 24μl
1.0μl	Exonuclease-free Klenow fragment of DNA Polymerase I
25μl	Final Volume

- Gently Mix contents of tube
6. Incubate tube at 37C for 1 hour.
 7. Stop the reaction by adding 1 μ l of 0.5M EDTA.
 8. Store probe at -20C until use (but try to use ASAP)

V. Prehybridization and Hybridization with QuikHyb Kit

*** the membrane should be covered with a thin film of QuikHyb solution at all times**

1. Prior to prehybridization, briefly dip the Nylon + membrane in deionized water to remove excess salt.
2. Add a minimum of 10ml of QuikHyb solution to the Nylon + membrane inside the roller bottles. Prehybridize at 65C for 10 minutes. Add salmon sperm DNA and prehybridize for another 10 minutes.
3. Boil 12 μ l of the double-stranded probe and 100 μ l of the salmon sperm DNA for 2 minutes. Remove 1ml of the prehybridization solution from the glass tube and mix with probe. This is enough probe to hybridize with 2 filters.
4. Place this mixture back in the glass tube and hybridize in QuikHyb solution at 65C for 2 hours.

VI. Wash

1. Prepare ahead 1L 2x SSC, 0.1% SDS; 1L 1x SSC, 0.1% SDS; and 1L of 0.1x SSC, 0.1% SDS. Warm last two solutions to 65C before beginning. Prewarm the Bellco Hot shaker water bath to 60C.
2. Place Hyb tubes in rack behind plexiglass work area. Wrap a kimwipe around Hyb tube cap to catch condensation while opening tube. Drain liquid into ³²P waste container, using kimwipe to stop any drips from rolling down the outside of tube.
3. Discard kimwipe into ³²P waste container. Pour 50ml of 2x SSC, 0.1% SDS into the tube, mix and drain liquid into ³²P waste container.
4. Pour 50ml of 2x SSC, 0.1% SDS into the tube and return to the Hyb oven for ~15minutes. Repeat for another 15 minute wash.
5. Place 200-500ml of 1xSSC, 0.1% SDS into a flat rectangle Rubbermaid container behind the screen. Pour off wash solution from tube as described in 3. Use forceps to pull filters out of the tube and into the Rubbermaid container. Filters from other tubes can be combined into the same container.
6. Place lid onto container and float in Hot Shaker water bath for 30 minutes.
7. Pour off liquid from Rubbermaid container into labeled beaker. Check counts of used wash solution in beaker. If higher than 200DPM, place in ³²P waste.
8. Check counts of filters individually by placing individually on blotting papers on the bench. If in the 400-500 DPM or lower, blot between kimwipes and place between plastic sheet protector without allowing to fully dry. If above 500 DPM, continue high stringency washes.

VII. Autoradiography

1. Arrange and trim page protectors to fit film holder containing 2 intensifier screens. Load Kodak X OMAT film in the darm room 227 as shown below and expose film in the -80 freezer for 2-4 days.
2.

	intensifier screen
	film
	filter sandwiched in page protector
	intensifier screen
3. Expose screen in X-ray developer room located in 34B Gourle Hall.
4. Turn on switch on X-ray developer and press "Run". This will cycle new fluids into the machine. Wait 15-20 minutes before adding film.
5. H₂O hose (white and clear tube) should be on 1/2 turn.
6. Laves knobs on "Close".
7. When the "Ready" light is on, put in X-ray film and close top of machine. Film will feed in automatically, it does not matter which side is up, and you can handle the film immediately afterwards.
8. The first film to be fed through should be a large exposed film (black in color) in order to clean the machine. After this film has been fed through, start to load samples.

Pulse-Field Gel Electrophoresis (PFGE) Protocol for TAC library

I. Plasmid Prep

1. Perform a plasmid prep for the TAC clone of interest using 1.8ml of Terrific broth containing 20-25 μ g/ml kanamycin for E. coli culture. Do miniprep using Maniatis protocol, the DNA is dissolved in 30 μ l TE/RnaseA. May add IPTG to increase plasmid copy number per cell.

II. Restriction Digest to Release TAC insert

1. Digest 2-5 μ l of the DNA from (I) with 0.1 μ l (1unit) of I-SceI (in a 20 μ l volume) and digest 5 hours or overnight at 37C.
2. *note that Walid Hamada uses 10 μ l of DNA with an alkaline lysis prep and you can also do a double digest with SFI and NotI to release insert. But if using SFI and NotI, digests must be performed at separate times (NotI first, then SFI) because the two enzymes are not compatible and the salt/buffer concentration must be optimized after the first digest.

III. PFGE – Selby Hall rm 202 (manual in glass cabinet)

1. Drain previous buffer out of PFGE machine by attaching tubing to drain hole.
2. Add new 1 x TAE buffer directly to PFGE main apparatus. Takes 2L.
3. Turn on cooler 2+ hours before use and make sure pump is working (ie buffer flowing through tubing). Set cooler to 14C.
4. Turn on Chef-Dr III system. Press initial switch time button and adjust to 5 seconds. Press final switch time button and adjust to 15 seconds. Press run time button and set to 16 hours. Press volt/cm and set to 4.5 hours. Included angle should always be set to 120 degrees.
5. **Make sure that all blocks except block 1 are set to zero for initial/final switch time and volts/cm.** There are three blocks total.
6. Gel casting system should be either in room 202 under the PFGE machine or with Chang in room 231. Place black flat gel holder in clear apparatus, tighten clamps surrounding casting apparatus.
7. Make up 1% PFGE gel with PFGE agarose from BIORAD. 1.1g agarose in 110ml of 1x TAE. After somewhat cool, pour agarose into gel cast, allow the gel to set. Take the gel out of the gel cast and place into PFGE machine with the black gel holder attached.
8. Load entire sample into wells using standard loading dye.
9. Run two ladders (λ Hind III and λ from NEB already in an agarose plug).
10. Press start on the Chef-Dr III system.
11. After running gel, stain in 500ml of 1x TAE (10 μ l of 10mg/ml EtBr) for 30 minutes. Rinse briefly and visualize with CCD camera in Selby rm 231.