Molecular Biology
Protocols for Aquatic Organisms

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Beth received her undergraduate degree in Biology at Centro Escolar University and pursued her Master of Science in Biology at the University of the Philippines in Diliman. She also worked as Research Associate at the Marine Science Institute before obtaining her Ph.D degree at Tokyo University of Marine Science and Technology. She is now an Associate Professor at the Department of Biological Sciences, College of Science and researcher at the Molecular Biology and Biotechnology Laboratory, Research Center for the Natural and Applied Sciences, University of Santo Tomas. She is currently teaching undergraduate and graduate courses such as Current Techniques in Molecular Biosciences, Marine Biotechnology, Ecology, Functional Genomics and
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Chapter I: Working with DNA/RNA

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Polymerase chain reaction (PCR) – Standard or colony

Materials

- Distilled or deionized H₂O (sterile)
- 10X PCR buffer
- dNTPs (2 mM)
- Taq polymerase
- Primer A
- Primer B
- DNA template (dissolved DNA or bacterial colony)
- MgCl₂ (if this is not included in the PCR buffer)

Methods

1. Mix the reagents in a PCR tube to make a 30 µl reaction per sample: (note: volume of reagents can be changed according to the reaction requirement).

Correspondence/Reprint request: Dr. Mary Beth I. Bacano-Maningas, University of Santo Tomas, Manila, Philippines. E-mail. mbmaningas@mnl.ust.edu.ph
Distilled/Deionized H₂O (sterile) - 21.0 µl
10X PCR buffer with MgCl₂ - 3.0 µl
dNTPs (2 mM) - 2.5 µl
Taq polymerase - 0.5 µl
Primer A - 1.0 µl
Primer B - 1.0 µl
DNA template - 1.0 µl of dissolved DNA for standard PCR or 1 bacterial colony for colony PCR

2. Run in a PCR machine with the following conditions: (note: conditions can be changed according to the reaction requirement).

Initial denaturation 95°C – 5 min.
30 cycles of denaturation 95°C – 30 sec
  * annealing 55°C – 30 sec
  * extension 72°C – 1 min
Final extension 72°C – 5 min
Final storage 4°C

* annealing temperature will vary based on the GC content or annealing temperature of the set of primers used
* extension time should be adjusted based on the target size. Ideally for 0.5 kb=30 S, 1Kb=1 min

3. Run in 1% (1 g in 100 ml) agarose gel with ethidium bromide (1-2 ul of 10mg/ml stock solution) at 100 V at about 20 to 30 min.
4. Observe red fluorescent bands using UV light (in Densitograph machine).
5. Print or save picture.

**Gel electrophoresis**

**A. Agarose gel electrophoresis**

**Materials**

<table>
<thead>
<tr>
<th>Agarose gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TBE (890 mM Tris-borate, 890 mM boric acid, 20mM EDTA)</td>
</tr>
<tr>
<td>Ethidium bromide (10mg/ml)</td>
</tr>
</tbody>
</table>
Methods

A. Gel preparation

1. To make a 1% agarose gel, weigh 1 g of agarose,
2. Add this to 100 ml of 1X TBE.
3. Boil in microwave until agarose is fully dissolved.
4. Add 2 μl of Ethidium bromide (10mg/ml).
5. Mix thoroughly.
6. Pour in agarose gel receptacle and place comb as in picture.

7. Stand until gel solidifies (note: for faster solidification, put in 4°C refrigerator).
8. Gel could be stored for sometime at 4°C refrigerator flooded with 1xTBE buffer wrapped in Saran wrap to prevent drying.

B. Pulse-field gel electrophoresis (PFGE)

Materials

- 0.5X TBE
- Pulse-field certified agarose
- Ethidium bromide
- Glass flask (specific for PFGE)
- Container

Methods

A. Preparing the receptacle

1. Tilt drain receptacle using hose and bucket.
2. Drain remaining liquid in the machine by turning on pump and raising the hose that is connected to the cooling module.
3. Put 2 L dH₂O then clean/ scrape with hands.
5. Put cover for holes.
6. Add 2L of 0.5X TBE.
7. Connect hose from the cooling module.
8. Turn on pump, set to 70-80 then allow circulation of buffer.
9. Turn on cooling module.
10. Set temperature, use raise button and set to 14°C.
12. Wait to cool down.

B. Gel preparation

1. Wash the gel set-up with tap H₂O (note: use gloves to prevent DNA contamination).
2. Rinse with dH₂O.
3. Wipe with paper towels.
4. Assemble gel set-up as shown below. Comb should have a clearance of 2-3 mm from the base.

![Pulse-field gel set-up](image)

5. Prepare pulse-field agarose gel as follows:
   - rinse flask with dH₂O
   - put 150 ml 0.5X TBE
   - put 1.5 g pulse-field certified agarose directly to flask (note: to dissolve easily, add and mix gel in a step wise manner at 0.3-0.5 g interval)
   - boil in microwave,
   - pour into gel set-up,
   - use tip to release/remove bubbles,
   - allow to dry for 30 min.

6. Put dH₂O before removing the comb from the gel.
7. Set square plate in the receptacle then place gel on steel plate.
8. Put 5 µL of 10X loading buffer to each 20 µl restriction mixture.
9. Load sample as shown in example below:
10. Turn on control panel.
11. Set the following settings:
   (Block = B1, Volts = 6, Run = 7 hrs., Initial SW = 1, Final SW = 5)
12. Press “start”.
13. Check for the presence of bubbles.
14. Label (name, date and inclusive time of running).
15. Put 0.5X TBE in plastic container.
16. Add 2-3 µL Ethidium bromide for every 100 ml of buffer.
17. Turn off control module.
18. Turn off cooling module then pump.
19. Put gel in container with 0.5X TBE and ethidium bromide.
20. Place in dark place for 30 min.

C. Gel reading

1. Put gel in dH₂O for 30 min after incubating in the dark room with ethidium bromide.
2. View in Densitograph (note: avoid prolonged exposure of DNA to UV).
3. Picture gel with a UV ruler and putting the 0-value parallel to wells on the gel.

Cloning with T-vector

Materials

LB Broth medium

For 100 ml, mix the following reagents:
- Tryptone = 1.0 g
- Yeast = 0.5 g
- NaCl = 0.5 g
Adjust to pH 7 with 5 N NaOH (optional)
Add deionized H₂O to a final volume of 100 ml Autoclave.
LB agar

For 100 ml mix the following reagents:
- Tryptone = 1.0 g
- Yeast = 0.5 g
- NaCl = 0.5 g
- Agar = 1.5 g
Adjust to pH 7 with 5 N NaOH (optional)
Add deionized H2O to a final volume of 100 ml
Autoclave
Put in 50°C incubator before use.

2xYT medium

For 100 ml mix the following reagents:
- Tryptone = 1.6 g
- Yeast = 1.0 g
- NaCl = 0.5 g
Adjust to pH 7 with 5 N NaOH (optional)
Add deionized H2O to a final volume of 100 ml
Autoclave.

LB-Ampicillin

1. Mix the following reagents (for 100 ml): (note: after autoclaving, cool down Drug Free LB Broth to about 50°C before adding antibiotic to prevent the latter degradation due to high temperature),
   - Drug Free LB Broth, autoclaved = 100 ml
   - 10 mg/ml Ampicillin = 100 μl
2. Mix gently.

LB-Ampicillin /X-gal/IPTG agar plates for blue-white colony selection

1. Mix the following reagents (note: after autoclaving, cool down LB Agar to about 50°C before adding antibiotic to prevent the latter degradation due to high temperature),
   - LB Agar, = 400 ml
   - Ampicillin = 400 μl
   - X-gal = 200 μl
   - IPTG = 80 μl
2. Mix gently then pour to Petri dishes, about 15-20 ml for every Petri dish.
3. Swirl gently to spread out the broth mixture in the dish.
4. Cool and dry for 5-15 min at room temperature (note: petri dishes should be turned upside down).
5. Wrap with Saran wrap then store at 4°C.
Methods

A. PCR Amplification of target DNA

1. Amplify target DNA using designed specific forward and reverse primers.
2. Set up the following 30 µl PCR reactions:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O (sterile)</td>
<td>21.45 µl</td>
</tr>
<tr>
<td>10X exTaq Buffer</td>
<td>3.00 µl</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>2.50 µl</td>
</tr>
<tr>
<td>ex Taq</td>
<td>0.05 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.00 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.00 µl</td>
</tr>
<tr>
<td>DNA Template</td>
<td>1.00 µl</td>
</tr>
</tbody>
</table>

3. Use the following PCR conditions:
   - 95°C for 5 min
   - 95°C for 30 sec; 55°C for 30 sec; 72°C for 1.5 min (30 cycles)
   - 72°C for 5 min
   - 4°C for storage until electrophoresis
4. Run in 1% Agarose gel.

B. Purification of PCR fragment

1. Cut agarose gel containing DNA as shown by U.V. light and place in 1.5 ml microfuge tube labeled accordingly.
2. Weigh cut agarose gel.
3. Add 3X volume of NaI and 0.5 volume of Melting Buffer (Toyobo Purification Kit), example: 100 µg of gel put 300 µl NaI and 50 µl of Melting Buffer.
4. Incubate for 10 min at 50°C.
5. Add 5 µl silica milk (Toyobo Purification Kit) to mixture.
6. Mix well (vortex or finger flick) and incubate for 5 min on ice.
7. Spin down for about 5 sec at 9,000 rpm and then pipette out supernatant (leave pellet).
8. Perform the following washing for 3X: (note: leave a small amount of supernatant on the last washing step then pipette out)
   - add 500 µl of Toyobo Washing Buffer
   - dissolve pellet by vortexing
   - spin down for 5 sec. at 9,000 rpm
   - decant supernatant
1. Dissolve in 5 to 10 µl of distilled H₂O.
2. Incubate for 3 min at 45°C – 55°C using hot water and thermometer.
3. Spin down for 2 min at 120,00 rpm.
4. Transfer supernatant to clean microfuge tube.
5. Store at -20°C.

C. Ligation of PCR fragment to T easy vector

1. Briefly centrifuge the T-Vector Easy and collect contents at the bottom of the tube.
2. Vortex the 2X Rapid Ligation Buffer vigorously before each use.
3. Mix the following reagents:

   2X Rapid Ligation Buffer, T4 DNA Ligase - 2.5 µl
   pGEM T Easy Vector - 0.5 µl
   PCR product - 1.5 or 2.0 µl
   T4 DNA ligase (4 Weiss U/ul) - 0.5 µl
   - ddH20 to a final volume - 5.0 µl


D. Transformation of competent cells with ligated PCR product

1. Mix 5 µl of ligated PCR product to 50 µl competent cells (DH5a or JM109) in microfuge tubes labeled appropriately,
2. Stand on ice for 30 min.
3. Heat shock the cells at 42°C for 45 sec to 1 min, then stand on ice for 2 min.
4. Add 500 µl of Drug Free LB or SOC Broth,
5. Cover microfuge tubes with parafilm.
6. Put in H₂O bath for 1 hr. with shaking at 37°C,
7. Centrifuge mixture for about 2 min at 6,000 rpm.
8. Remove half of the supernatant to concentrate mixture then dissolve pellet using pipette.
10. Incubate for overnight at 37°C and check for presence of bacterial colonies the next day.

E. Screening and culture of transformed competent cell

1. Select 3- 5 white colonies and streak in new LB-Ampicillin /X-gal/IPTG agar plates.
2. Incubate for overnight at 37°C.
3. Transfer 1-2 white colonies to test tubes containing 2 ml of LB-Ampicillin (use sterile conditions).
4. Place culture mixture to shaker-incubator for overnight at 37°C.

**F. Do plasmid extraction (refer to section on plasmid extraction)**

**H. Checking of DNA insert**

**Enzyme restriction method:**

1. Mix the following reagents to make a 10 ul reaction:

   - EcoR1 = 0.5 μl
   - 10 x H Buffer = 2.0 μl
   - dH₂O = 5.5 μl
   - Plasmid = 2.0 μl

2. After mixing, dispense to each sample tube containing 2 μl of plasmid.
3. Spin down for 5 sec at 120 rpm.
4. Incubate for 2 h at 37°C.
5. Run in 2% agarose gel to check for the presence of the insert.

**PCR method**

1. Run a standard PCR using the specific primers for the insert and the extracted plasmid as template.
2. Run in 2% agarose gel to check insert.

**Plasmid extraction and purification**

1. **Small scale extraction**

   **Materials**

   Solution I (for 200 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.8 g</td>
</tr>
<tr>
<td>1 M Tris-HCl</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.74 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Solution IIA (for 100 ml)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>- 1.6 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>- 100 ml</td>
</tr>
</tbody>
</table>

Solution IIB (for 100 ml)

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>- 2.0 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>- 100 ml</td>
</tr>
</tbody>
</table>

Solution III (for 500 ml)

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KOaC</td>
<td>- 147 g</td>
</tr>
<tr>
<td>glCH₃COOH</td>
<td>- 57.5 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>- 500 ml</td>
</tr>
</tbody>
</table>

Phenol-Chloroform Isoamyl (PCI)
95% Ethanol
75% Ethanol
RNase
1X TE Buffer
20% PEG with 2.5 M NaCl

**Manual extraction**

1. Transfer by pouring 1.5 ml of transformed competent cells to Eppendorf tubes.
2. Centrifuge at 6,000 rpm for 2 min at 4°C.
3. Aspirate the supernatant and keep cell pellet.
4. Add 100 μl of Solution I and then vortex to resuspend pellet.
5. Add 100 μl of Solution IIA (2% SDS) and 100 μl of Solution IIB (0.4 N NaOH), then mix gently.
6. Add 150 μl Solution III and then mix vigorously.
7. Stand on ice for about 5 min.
8. Centrifuge at 13,000 rpm at 4°C for 5 to 10 min.
9. Transfer supernatant into a fresh microfuge tube.
10. Add 400 µl of Phenol-Chloroform-Isoamyl alcohol.
11. Mix vigorously more than 50 – 100 times.
12. Centrifuge at 13,000 rpm at 4°C for 5 min.
13. Transfer supernatant (containing Plasmid) to a new microfuge tube.
14. Add 1 ml of cold 99% Ethanol then mix vigorously (finger-flick).
15. Incubate mixture at room temperature for 5 min.
16. Centrifuge at 13,000 rpm at 4°C for 10 min.
17. Aspirate 99% Ethanol.
18. Dry at room temperature for about 30 min.
19. Add 50 µl of distilled H20 and 2 µl of RNase then spin down.
20. Incubate at 37°C for 1 hr.
21. Add 35 µl of 20% PEG and 2.5 M NaCl to DNA sample, mix well then spin down.
22. Stand on ice for 30 min.
23. Centrifuge at 12,500 rpm at 4°C for 10 min.
25. Add 500 µl of 75% ethanol.
26. Centrifuge at 13,000 rpm at 4°C for 5 min.
27. Discard supernatant by aspiration.
28. Centrifuge again in order to discard absolutely supernatant.
29. Dry up tubes at room temperature (note: tubes turned upside down on Kimwipes).
30. Add 50 µl of 1x TE Buffer.
31. Store at -20°C until use.

2. Large scale extraction

Materials

Beckman tubes with cap
10 µg/ml Ethidium bromide
Saturated isopropanol
Dialysis bag (boiled for 10 min)
3 L of 1X TE
Cesium chloride (CsCl)

Methods

A. Centrifugation

1. Mix 3.7 to 3.8 g CsCl to 4 ml samples each placed in 15 ml corning tube. (note: dissolve completely).
2. In Beckman tube, put 175 to 200 μl of 10 μg/ml of ethidium bromide.
3. Put CsCl-sample to Beckman tube with ethidium bromide.
   3.1. Put 1 ml first and rotate the tube to wet its sides
   3.2. Carefully add the rest of the CsCl-sample mixture, avoiding the formation of bubble(s)
4. Place black cap to seal the Beckman tube then mix-invert the solution.
5. Prepare for ultracentrifugation:
   5.1. Select the rotor wells where the tubes could easily be put in and taken out
   5.2. Put the tubes then cover with gold cap
   5.3. Put black rotor cap and tighten
   5.4. Place rotor in ultracentrifuge machine and then close
   5.5. Set the machine to the following:
   - 65,000 rpm
   - 6 to 12 hrs
   - 22°C
   - acceleration (max); deceleration (max)
6. Start machine, wait until maximum speed to check if it runs smoothly.
7. After running, press vacuum before opening.
8. Remove the rotor from the machine, then the rotor cap then the gold cap.
9. Put tubes in the black rack as shown:

10. View DNA bands using UV light in the darkroom.
11. Use a syringe to extract the DNA as shown. Place the needle just below the lower line of the band, pierce then extract.
12. Discard waste in ethidium bromide bottle.
13. Mix extract with saturated isopropanol. Pipette out upper layer with ethidium bromide then put isopropanol. Do this again until lower layer becomes clear.
14. Dialyze the cleared lower layer.

**B. Dialysis**

1. Close dialysis bags on one end using clips (orange or green). Fold 3X before Clipping.
2. Load the purified DNA to the dialysis bag using a pipette.
3. Close the other end of the bag also using clips. Fold 3X before clipping.
4. Put in autoclaved 3 L 1X TE and dialyze for 24 hrs or more.
5. After dialysis, transfer liquid inside the bag to 15 ml corning tube.
Polymerase chain reaction-random amplification of cDNA Ends (PCR-RACE) - SMART™

Materials:

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>5’-CDS primer</th>
<th>3’CDS primer A</th>
<th>SMART II A oligo</th>
<th>5X First-Strand Buffer</th>
<th>DTT (20mM)</th>
<th>dNTP Mix (10mM)</th>
<th>PowerScript Reverse Transcriptase</th>
</tr>
</thead>
</table>

Methods

1. First-strand cDNA synthesis

1. Make 10 µl reactions for each of the following in microcentrifuge tubes:

5’RACE-ready cDNA
   - 1-3 µl RNA sample
   - 1 µl 5’ CDS primer
   - 1 µl SMART II A oligo

3’ RACE-ready cDNA
   - 1-3 µl RNA sample
   - 1 µl 3’ CDS primer

2. Put DW to a final volume of 5 µl for each reaction.
3. Mix the 2 tubes and spin briefly.
4. Incubate at 70°C for 2 min, then quickly put on ice for 2 min.
5. Spin tubes briefly to collect contents.
6. Add the following to make 10 µl total volume reaction:

   2 µl 5X First-Strand Buffer
   1 µl DTT (20 mM)
   1 µl dNTP mix (10 mM)
   1 µl Reverse Transcriptase

7. Mix then spin down tube.
8. Incubate at 42°C for 1.5 hr in an air incubator.
9. Dilute with Tricine-EDTA buffer:
   - add 10 µl if started with <200 ng total RNA
   - add 100 µl if started with >200 ng total RNA
   - add 250 µl if started with poly A+ RNA

10. Heat tubes at 72°C for 7 min.
11. Store samples at -20°C for up to 3 mos.

2. PCR

1. Proceed to regular PCR using specific and universal primers and the 1st strand cDNA as template.

Polymerase chain reaction- Reverse transcription (PCR-RT)

1. Total RNA extraction

**Materials**

<table>
<thead>
<tr>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizol</td>
</tr>
<tr>
<td>Chloroform</td>
</tr>
<tr>
<td>Isopropanol</td>
</tr>
<tr>
<td>75% DEPC Ethanol</td>
</tr>
<tr>
<td>DEPC H₂O</td>
</tr>
<tr>
<td>Glass homogenizer</td>
</tr>
</tbody>
</table>

**Methods**

1. Dissect fish and take out desired tissues.
2. Add Trizol *(note: amount of tissue should be less than 10% of the total volume of Trizol)*.
3. Homogenize using glass homogenizer and syringe.
4. Add 200 ul of 100% chloroform.
5. Shake vigorously for 15 sec.
6. Stand at room temperature for 5 min.
7. Centrifuge at 12,000 rpm at 4°C for 25 min.
8. Pipette out supernatant, *(if supernatant is milky, do step 2 to 8 again).*
10. Invert/mix gently.
12. Stand for 10 min at room temperature.
13. Centrifuge at 12,500 rpm at 4°C for 20 min.
15. Put 1 ml of DEPC-treated 75% ethanol.
16. Centrifuge at 12,500 rpm at 4°C for 15 min.
17. Decant supernatant.
18. Put 16 ml of DEPC treated H2O.

2. cDNA synthesis

Materials:

- RNA sample
- Poly DT primer
- dNTP mix
- 5X First Strand buffer
- DTT (Dithiothritol),
- RNase inhibitor,
- MMLV reverse transcription enzyme,

Methods

1. Mix the following:
   - 16 μl of RNA sample
   - 1 μl of Poly DT primer
   - 1 μl of 10 mM dNTP mix

2. Mix gently by finger-flicking.
3. Anneal for 5 min at 65°C.
4. Put on ice immediately after reaction.
5. Add the following to the annealed sample:
   - 4 μl of 5X First Strand buffer
   - 2 μl of DTT (Dithiothritol),
   - 0.25 μl of RNase inhibitor,
   - 0.50 μl of MMLV reverse transcription enzyme

6. Finger-flick then spin down.
7. Incubate at 37°C for 50 min.
8. Heat at 70°C for 15 min.
9. Maintain at 4°C.
3. Run in PCR

1. Use primers and β-actin for testing if reverse transcription was successful.
2. Measure DNA concentration then dilute to normalize all samples.
3. Run PCR with normal settings.

Southern hybridization

Materials:

| HCl     | NaOH     | Plastic tray | Plastic plates | Kim towel | 3M paper | Hybond N+ membrane |

Methods

a. DNA transfer (Southern blot)

1. Soak gel in 200 ml of 0.25 N HCl using plastic box for 20 min.
2. Transfer gel to another plastic tray containing 200 ml 0.4 NaOH and soak for 20 min.
3. Prepare preliminary Southern blot set-up as shown (top view):
4. Put 300 ml 0.4 NaOH in plastic container and place 3M paper touching the liquid.
5. Remove bubbles in the space between plastic plate and the 3M paper.
6. Place gel on top of the 3M paper with the wells facing down.
7. Remove bubbles in between 3M paper and gel using 0.4 NaOH.
8. Put folded Saran wrap on the sides of the gel as shown (top view):

9. Put membrane (Hybond N+) previously soaked in NaOH (make sure no bubbles are present in between membrane and the gel).
10. Put 3 pcs. of 3M membrane previously soaked in NaOH.
12. Place the glass plate then put weight approximately 500 g,
13. Final Southern blot set-up is shown below:

B. Post-southern blot procedure

1. Remove bottle.
2. Remove paper towels then the 3M paper.
3. Remove Saran wrap.
4. Invert-transfer gel with membrane (Hybond N+) in the table with Saran wrap. Hybond N+ is now in the bottom.
5. Mark “well” position using ball point pen up to gel loaded.
6. Soak in 2X SSC for 30 sec; shake a little on the table.
7. Place in 3M paper twice the size of the membrane.
8. Fold 3M paper to cover membrane.
9. Incubate at 80°C for 2 hrs.

C. RI labeling and processing

1. Proceed to RI labeling similar to procedure in BAC library screening

**cDNA library screening**

**Materials**

**A. Probe mixture (6 μl)**

1. Mix the following in a 0.6 ml tube:
   - autoclaved dH₂O = 4 μl
   - DNA probe = 2 μl

**B. Hybridization Buffer (200 ml)**

(Note: Clean the glasswares thoroughly with dishwashing soap and brush, and rinsed with distilled H₂O. Cover with Saran wrap before use)

1. Mix the following reagents using a 200 ml cylinder:
   - 20X SSC = 50.0 ml
   - dH₂O = 135.0 ml
   - 100X Denhardt’s solution = 10.0 ml
   - 20% SDS = 5.0 ml

2. Mix chemicals in the proper order.
3. Dissolve and raise volume to 200 ml with dH₂O if necessary.
4. Swirl or mix gently.
5. Cover with Saran wrap.
6. Immediately incubate in H₂O bath for 2 min. at 37°C to 40°C.
7. Filter with 0.22 μm micropore.
8. Keep in H₂O bath (37°C to 40°C) before use.
C. Hybridization Buffer (50 ml)

1. Mix the following reagents using a 100 ml cylinder:
   - 20X SSC = 12.50 ml
   - dH2O = 33.75 ml
   - 100X Denhardt’s solution = 2.50 ml
   - 20% SDS = 1.25 ml

2. Mix chemicals in the said order.
3. Dissolve and raise volume to 50 ml with dH2O if necessary.
4. Swirl or mix gently.
5. Cover with Saran wrap.
6. Incubate in H2O bath for 2 min. at 37°C to 40°C.
7. Filter with 0.22 μm micropore.
8. Keep in H2O bath (37°C to 40°C) before use.

D. Wash Buffer

(Note: Add SSC and dH2O before adding SDS then raise to final volume with dH2O to prevent precipitation)

(400 ml) (100 ml)

1. 2X SSC, 0.1 % SDS
2. 2X SSC, 0.1 % SDS

- 20X SSC = 40.0 ml
- 20X SSC = 10.0 ml
- 20% SDS = 2.0 ml
- 20% SDS = 0.5 ml
- raise to 400 ml dH2O
- raise to 100 ml dH2O

3. 0.1X SSC, 0.1 % SDS
3. 0.1X SSC, 0.1 % SDS

- 20X SSC = 20.0 ml
- 20X SSC = 5.0 ml
- 20% SDS = 2.0 ml
- 20% SDS = 0.5 ml
- raise to 400 ml dH2O
- raise to 100 ml dH2O

F. Denaturing and neutralizing buffer

1. Denaturing buffer (0.2 N NaOH and 1.5 M NaCl)

- 20X SSC = 2.0 ml
- 20X SSC = 0.5 ml
- 20% SDS = 2.0 ml
- 20% SDS = 0.5 ml
- raise to 400 ml dH2O
- raise to 100 ml dH2O
2. Neutralizing buffer (0.4 M Tris-HCl and 2X SSC)

- 2 M Tris-HCl, pH 7.2-7.5 = 20.0 ml
- 20X SSC = 10.0 ml
- raise to 100 ml using dH₂O

3. 2X SSC

- 20X SSC = 20.0 ml
- raise to 200 ml using dH₂O

G. Other solutions

(Note: Mix HCl and NaOH in glass)

1. 100X Denhardt’s Solution (100 ml)

- Ficoll = 2.0 g
- Polyvinylpyrrolidone = 2.0 g
- Bovine Serum Albumin = 2.0 g
- raise to 100 ml dH₂O

1.1. Mix well in mortar and pestle (note: very important)

2. 20X SSC (2 L)

- Sodium Chloride (NaCl) = 350.6 g
- Sodium Citrate = 176.4 g
- raise to 2 L using dH₂O

3. 5M NaCl (1L):

- NaCl = 292.2 g
- raise to 1.0 L using dH₂O

4. 0.25 N HCl (200 ml):

- Concentrated HCl = 4.0 ml
- dH₂O = 196.0 ml

5. 0.4 N NaOH (500 ml)

- NaOH = 8.0 g
- raise to 500 ml using dH₂O
Methods

(Note: While in the Radioisotope Room always log in and observe safety procedures. Wear protective gears e.g. gloves, slippers, lab gown, RI meter etc. and be sure to carry a cell phone in case of emergency. Be cautious and avoid PANICKING)

A. Membrane preparation

1. Culture colonies on plates.

3. Blot the membrane over the plate.
4. Incubate in oven for 2 hrs at 80°C.

B. Radioisotope processing

1. Hybridization

1. Turn on incubator/ shaker and set to 65°C.
2. Lay membrane on paper towel using forceps.
3. Put membrane inside the plastic bag.
4. Add hybridization buffer.
5. Remove the bubbles and make sure that the membrane is soaked completely.
7. Soak membrane for about 1 hr at 65°C and shake in the incubator/shaker for 20 min. (note: heat up hybridization buffer to 65°C while performing pre-hybridization)

2. Probe labeling

1. On the PCR machine run file 777 (note: use gloves).
2. Add 1 µl Random primer (#1) to DNA Probe mixture in 0.6 ml tubes.
3. Mix then centrifuge.
4. Run mixture at the PCR machine for 3 min at 95°C (Step 1).
5. Get Radioisotope P$_{32}$ from the refrigerator at the Radioactive room.
7. Add 1.25 µl of 10X Buffer (#2) and 1.25 ul of dNTP (#3).
8. Add 0.5 µl of Klenow fragment solution (#4).
9. Put 2.5 µl of P$_{32}$ to the Probe labeling solution (note: 1 – 14 day old P 32 use 2.5-3 ul, after 14 days use about 4 ul) do this carefully at the hood because RI is very dangerous.
10. Run mixture at PCR machine 37°C for 15 min and then at 95°C for 3 min (Step 3 and 4).
11. Put on ice until membrane labeling/ hybridization.

3. Membrane labeling/ hybridization

1. Cut the edge of the pre-hybridized plastic bag with membrane.
2. Add 3 - 4 µl of Labeling probe solution to the plastic bag (note: add probe away from the membrane).
3. Put in incubator/shaker for at least 12 hrs. at 65°C. (note: for same species probe use 65°C and for degenerate probes use lower temperature).

4. Membrane washing

(Note: Don`t allow membrane to dry during this step)

1. Prepare Wash Buffer E.1 (400 ml) in a 500 ml beaker.
2. Heat to 65°C at the stove using thermometer.
3. Put in plastic box with labeled membrane (note: discard first the existing solution to the black container near the refrigerator)
4. Incubate with shaking at incubator/shaker for 5 min.
5. Discard wash buffer in black container.
6. Prepare Wash Buffer E.2 (400 ml) in a 500 ml beaker.
7. Heat to 65°C at the stove using thermometer.
9. Incubate with shaking at incubator/shaker and for 5 min..
10. Discard wash buffer again in black container.
11. Prepare Wash Buffer E.3 (400 ml) in a 500 ml beaker.
12. Heat to 65°C at the stove using thermometer.
13. Put in plastic box with membrane.
15. Discard wash buffer again in black container.
5. Membrane drying

1. Lay paper towels.
2. Place membrane on top of paper towel and allow to dry completely

   (note: use forceps to pick-up membrane and check signal using RI survey meter).

6. Membrane packing

1. Cut a Saran wrap and place on top of BAS cassette (use as guide).
2. Put membrane over the Saran wrap taking note of the borders of the BAS cassette (Note: It is important to place membrane in the position showed below).

![Orientation of membrane in IP cartridge](image)

   Orientation of membrane in Computer

3. Lay over another piece of Saran wrap.
4. Fold cleanly and avoid touching membrane.
5. Place inside the cassette.
6. Put IP plate, white side facing downwards.
7. Put a paper towel on top of membrane.
8. Close BAS cassette and label with name.
9. Expose for at least 6 hours.

   (Note: Membrane in Saran wrap could be stored at -20°C if BAS cassette is unavailable. Processed membranes can be used ideally up to about 4 times. Use parafilm paper when discarding)

7. Membrane computer processing

1. Log on to the logbook.
2. Switch on the PC.
3. After PC is set, switch on the BAS machine (*note: check if the machine is closed*).
4. Wait for about 15 min for machine warm-up and the Ready signal is on.
5. Turn off the lights in the room.
6. Put incubated IP inside the BAS machine (*note: white side facing up*).
7. Push Start.
8. On the PC, open image reader icon.
9. Check “Read & Launch Image Gauge” is ok.
10. Type name then click on “read”.
11. Wait for a few minutes until reading is finished.
12. Save file to own folder.
13. Print to acetate using the following procedure: - click file, then print, then positioning button, then trimming, then to positioning again; when 100% scale is achieved, press print.
15. Wrap membrane in foil and store at -20°C.

*(Note: If IP is jammed, push clear. Wait for about 30 sec. to be discharged then open/close the lid, then, get the IP)*

**C. Positive colonies**

1. Culture positive colonies in appropriate selection antibiotic.
2. Do plasmid extraction.
3. Sequence.

**Bacterial artificial chromosome (BAC) library screening**

**Materials**

**A. Membrane**

*(Note: BAC membrane is already prepared from previous work)*

**B. Probe Mixture (6 ul)**

<table>
<thead>
<tr>
<th>1. Mix the following in a 0.6 ml tube:</th>
</tr>
</thead>
<tbody>
<tr>
<td>- autoclaved d H₂O = 4 µl</td>
</tr>
<tr>
<td>- DNA probe = 2 µl</td>
</tr>
</tbody>
</table>
C. Hybridization buffer (200 ml)

(Note: Clean the glasswares thoroughly with dishwashing soap and brush, and rinsed with distilled H$_2$O. Cover with Saran wrap before use)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>d H$_2$O</td>
<td>135.0 ml</td>
</tr>
<tr>
<td>100X Denhardt’s solution</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

1. Mix the following reagents using a 200 ml cylinder:

2. Mix chemicals in the proper order.
3. Dissolve and raise volume to 200 ml with dH$_2$O if necessary.
4. Swirl or mix gently.
5. Cover with Saran wrap.
6. Immediately incubate in H$_2$O bath for 2 min. at 37°C to 40°C.
7. Filter with 0.22 µm micropore.
8. Keep in H$_2$O bath (37°C to 40°C) before use.

D. Hybridization Buffer (50 ml)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>12.50 ml</td>
</tr>
<tr>
<td>d H$_2$O</td>
<td>33.75 ml</td>
</tr>
<tr>
<td>100X Denhardt’s solution</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>1.25 ml</td>
</tr>
</tbody>
</table>

1. Mix the following reagents using a 100 ml cylinder:

2. Mix chemicals in the said order.
3. Dissolve and scale up to 50 ml with dH$_2$O if necessary.
4. Swirl or mix gently.
5. Cover with Saran wrap.
6. Incubate in H$_2$O bath for 2 min. at 37°C to 40°C.
7. Filter with 0.22 µm micropore.
8. Keep in H$_2$O bath (37°C to 40°C) before use.
E. Wash buffer

(Note: Add SSC and dH₂O before adding SDS then raise to final volume with d H₂O to prevent precipitation)

<table>
<thead>
<tr>
<th>(400 ml)</th>
<th>(100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2X SSC, 0.1 % SDS</td>
<td>1. 2X SSC, 0.1 % SDS</td>
</tr>
<tr>
<td>- 20X SSC = 40.0 ml</td>
<td>- 20X SSC = 10.0 ml</td>
</tr>
<tr>
<td>- 20% SDS = 2.0 ml</td>
<td>- 20% SDS = 0.5 ml</td>
</tr>
<tr>
<td>- raise to 400 ml d H₂O</td>
<td>- raise to 100 ml d H₂O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. 1X SSC, 0.1 % SDS</th>
<th>2. 1X SSC, 0.1 % SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 20X SSC = 20.0 ml</td>
<td>- 20X SSC = 5.0 ml</td>
</tr>
<tr>
<td>- 20% SDS = 2.0 ml</td>
<td>- 20% SDS = 0.5 ml</td>
</tr>
<tr>
<td>- raise to 400 ml d H₂O</td>
<td>- raise to 100 ml d H₂O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. 0.1X SSC, 0.1 % SDS</th>
<th>3. 0.1X SSC, 0.1 % SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 20X SSC = 2.0 ml</td>
<td>- 20X SSC = 0.5 ml</td>
</tr>
<tr>
<td>- 20% SDS = 2.0 ml</td>
<td>- 20% SDS = 0.5 ml</td>
</tr>
<tr>
<td>- raise to 400 ml d H₂O</td>
<td>- raise to 100 ml d H₂O</td>
</tr>
</tbody>
</table>

F. Denaturing and neutralizing buffer

1. Denaturing buffer (0.2 N NaOH and 1.5 M NaCl)

| - NaOH = 0.4 g |
| - 5M NaCl = 15.0 ml |
| - raise to 50 ml using d H₂O |

2. Neutralizing buffer (0.4 M Tris-HCl and 2X SSC)

| - 2 M Tris-HCl, pH7.2-7.5 = 20.0 ml |
| - 20X SSC = 10.0 ml |
| - raise to 100 ml using d H₂O |
3. 2X SSC

- 20X SSC = 20.0 ml
- raise to 200 ml using d H₂O

G. Other solutions

(Note: Mix HCl in glass while NaOH in plastic)

1. 100X Denhardt’s Solution (100 ml)

- Ficoll = 2.0 g
- Polyvinylpyrrolidone = 2.0 g
- Bovine Serum Albumin = 2.0 g
- raise to 100 ml d H₂O

1.1. Mix well in mortar and pestle (note: very important).

2. 20X SSC (2 L)

- Sodium Chloride (NaCl) = 350.6 g
- Sodium Citrate (        ) = 176.4 g
- raise to 2 L using d H₂O

3. 5M NaCl (1L):

- NaCl = 292.2 g
- raise to 1.0 L using d H₂O

4. 0.25 N HCl (200 ml):

- Concentrated HCl = 4.0 ml
- d H₂O = 196.0 ml

5. 0.4 N NaOH (500 ml)

- NaOH = 8.0 g
- raise to 500 ml using d H₂O
Methods

A. First screening

(Note: While in the RI Room always log in and observe safety procedures. Wear protective gears e.g. gloves, slippers, lab gown, RI meter etc. and be sure to carry a cell phone in case of emergency. Be cautious and avoid PANICKING)

1. Hybridization

1. Turn on incubator/shaker and set to 65°C.
2. Lay membrane on paper towel using forceps.
3. Put membrane inside the plastic bag.
4. Add hybridization buffer.
5. Remove the bubbles and confirm that the membrane is soaked completely.
7. Soak membrane for about 1 hr at 65°C and shake in the incubator/shaker for 20 min (note: heat up hybridization buffer to 65°C while performing pre-hybridization).

Re-probe

1. Prepare fresh 200 ml of 0.1% Sodium Dodecyl Sulfide (SDS).
   1.1. Heat 200 ml of dH2O in the electric stove using the steel container.
   1.2. Put 1 ml of 20% SDS,
   1.3. Boil
2. Boil for about 2-3 minutes.
3. Take out membrane from plastic and throw garbage to the proper receptacle.
4. When SDS is boiling, put Membrane using fork (note: this procedure will remove the DNA from the surface of the membrane.)
5. Boil for about 2-3 minutes.

2. Probe labeling

1. On the PCR machine run file 777 (note: use gloves).
2. Add 1 ul Random primer (#1) to DNA Probe mixture in 0.6 ml tubes.
3. Mix then centrifuge.
4. Run mixture at the PCR machine for 3 min at 95°C (Step 1).
5. Get Radioisotope P$^{32}$ from the refrigerator at the Radioactive room.
7. Add 1.25 μl of 10X Buffer (#2) and 1.25 μl of dNTP (#3).
8. Add 0.5 μl of Klenow fragment solution (#4).
9. Put 2.5 μl of P$^{32}$ to the Probe labeling solution (note: 1 – 14 day old P$^{32}$ use 2.5-3 μl, after 14 days use about 4 ul) do this carefully at the hood because RI is very dangerous)
10. Run mixture at PCR machine 37°C for 15 min. and then at 95°C for 3 min. (Step 3 and 4).
11. Put on ice until membrane labeling/ hybridization.

3. Membrane labeling/ hybridization

1. Cut the edge of the pre-hybridized plastic bag with membrane.
2. Add 3 - 4 μl of Labeling probe solution to the plastic bag (note: add probe away from the membrane).
3. Put in incubator/shaker for at least 12 hrs at 65°C (note: for same species probe use 65°C and for degenerate probes use lower temperature).

4. Membrane washing

(Note: Don’t allow membrane to dry during this step)

1. Prepare Wash Buffer E.1 (400 ml) in a 500 ml beaker.
2. Heat to 65°C at the stove using thermometer.
3. Put in plastic box with labeled membrane (note: discard first the existing solution to the black container near the refrigerator).
4. Incubate with shaking at incubator/shaker for 5 min.
5. Discard wash buffer in black container.
6. Prepare Wash Buffer E.2 (400 ml) in a 500 ml beaker.
7. Heat to 65°C at the stove using thermometer.
9. Incubate with shaking at incubator/shaker and for 5 min.
10. Discard wash buffer again in black container.
11. Prepare Wash Buffer E.3 (400 ml) in a 500 ml beaker.
12. Heat to 65°C at the stove using thermometer.
13. Put in plastic box with membrane.
15. Discard wash buffer again in black container.
5. Membrane drying

1. Lay paper towels.
2. Place membrane on top of paper towel and allow to dry completely (note: use forceps to pick-up membrane and check signal using RI survey meter).

6. Membrane packing

1. Cut a Saran wrap and place on top of BAS cassette (use as guide).
2. Put membrane over the Saran wrap taking note of the borders of the BAS cassette (Note: It is important to place membrane in the position showed below):

   ![Diagram of membrane packing]

3. Lay over another piece of Saran wrap.
4. Fold cleanly and avoid touching membrane.
5. Place inside the cassette.
6. Put IP plate, white side facing downwards.
7. Put a paper towel on top of membrane.
8. Close BAS cassette and label with name.
9. Expose for at least 6 hours.

(Note: Membrane in Saran wrap could be stored at -20°C if BAS cassette is unavailable. Processed membranes can be used ideally up to about 4 times. Use parafilm paper when discarding)

7. Membrane computer processing

1. Log on to the logbook.
2. Switch on the PC.
3. After PC is set, switch on the BAS machine (*note: check if the machine is closed*).
4. Wait for about 15 min for machine warm-up and the Ready signal is on.
5. Turn off the lights in the room.
6. Put incubated IP inside the BAS machine (*note: white side facing up*).
7. Push Start.
8. On the PC, open image reader icon.
9. Check “Read & Launch Image Gauge” is ok.
10. Type name then click on “read”.
11. Wait for a few minutes until reading is finished.
12. Save file to own folder.
13. Print to acetate using the following procedure: click file, then print, then positioning button, then trimming, then to positioning again; when 100% scale is achieved, press print.
15. Wrap membrane in foil and store at -20°C.

(Note: If IP is jammed, push clear. Wait for about 30 sec. to be discharged then open/close the lid, then, get the IP)

8. Membrane reading

(Note: Please refer to figure on “How to read labeled membrane?”)

1. Take note of the membrane number (e.g. F29-121-128) and the positively labeled grids in it.
2. Determine the letter-number (e.g. 17-C) of the grid containing positives.
3. Check the position of the positives inside the grid, whether middle, upper left etc.
4. Afterwhich, determine the number tag of the positives, e.g. P123 (17-C) to be used in selecting the microtiter plates for second screening.

9. Microtiter plate screening

(Note: Refer to figure on Microtiter plate)

1. Pick up from Ultralow freezer the appropriate microtiter plates, e.g. for P123 (17-C) get Microtiter plate P123.
2. Pick up colony 17-C from the plate by first making a hole in the plastic cover using a heated paper clip and then picking up with sterilized toothpick (note: close the hole with sterilized tape after use).

3. Spread in a selective agar plate, labeled accordingly and culture overnight.

B. Second Screening

1. Membrane preparation

   1. Label the plate with culture as A1, A2 etc per section like in the figure:
2. Make membrane using Hybond – N+ (*note: make sure its N+ for DNA*) like the figure below, - make grid lines using the back edge of the cutter at 1 cm$^2$:

![Diagram of grid lines](image)

- cut out the sides of the square with a cutter,
- label membrane and grids using a ball point pen,
- write probe name at the back top of the membrane.
- cut the top left corner as shown to serve as marker

3. Cut out 3M paper twice the size of the membrane.
4. Soak 1 3M paper with denaturing buffer then place the membrane using forceps and soak for 5 min.
5. Transfer membrane using forceps to new 3M paper with neutralizing buffer and soak for 5 min. Do this twice.
6. Put membrane in 2X SSC.
7. Use plastic gloves when removing membrane.
8. Put on membrane on 3M paper (*note: 3M paper should be folded*).
8. Incubate in oven for 2 hrs at 80°C.

2. Colony hybridization

1. Do procedure similar to first screening except the following:
   - Use the existing probe left from First screening,
   - Since membrane is small, use the bottle not plastic bag,
   - Use 50 ml volume for the hybridization buffer,
   - Use 100 ml volume for the wash buffer,
   - Do only Steps 1-8
3. Culture of positive colonies

   1. Culture positively labeled colonies in 3 ml LB-chloramphenicol overnight in incubator-shaker.

4. BAC extraction (A protocol using the Qiagen Plasmid Kit was used)

   1. Put 800 ml of QF Elution Buffer in Eppendorf tube and incubate at 65°C until Use.
   2. Transfer overnight culture of positive colony to microfuge tube until full.
   3. Centrifuge at 4,000 rpm for 3 min.
   4. Aspirate supernatant (leave pellet on the tube).
   5. Put remaining culture in the same microfuge tube.
   6. Centrifuge at 4,000 rpm for 5 min.
   7. Aspirate supernatant (leave pellet on the tube).
   8. Put 400 µl of P1 Buffer (RNase + stock at 4°C-refrigerator).
   9. Resuspend pellet by vortexing.
   10. Add 400 µl of P2 Buffer.
   11. Mix 5-6 times gently.
   12. Stand at room temperature for 5 min.
   13. Add chilled P3 Buffer, mix immediately but gently.
   14. Stand on ice for 5 min.
   15. Centrifuge at 13,000 rpm at 4°C for 10 min.
   16. (Equilibrate column by adding 1 ml QBT Buffer and allowed to drip).
   17. Put supernatant on the equilibrated column.
   18. Wash with 1 ml QC Buffer 4X.
   19. Place clean microfuge tube in column after washing.
   20. Add 800 µl of QF Buffer.
   22. Mix gently.
   23. Centrifuge at 13,000 rpm for 30 min. at 4°C.
   24. Carefully aspirate supernatant using pipette.
   25. Add 700 µl of 75% ethanol.
   26. Centrifuge at 13,000 rpm for 5 min at 4°C.
   27. Aspirate supernatant using pipette.
   28. Dry/invert in tissue for 1-2 min.
   29. Resuspend in 20 µl dH2O.
   30. Incubate for 2 hrs at room temperature.
5. BAC DNA check

1. Run the extracts in 1% agarose to check for the presence of BAC DNA.
2. Take picture for future reference.

C. Third screening

1. Restriction set-up for southern blot

1. Restrict BAC DNA e.g. *Hind* III, *Eco*RI and/or *Pst*I
2. Do restriction in 0.6 ml tubes using the following mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAC DNA</td>
<td>6 µl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>1 µl</td>
</tr>
<tr>
<td>raise using dH2O to</td>
<td>11.5 µl</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20.0 µl</strong></td>
</tr>
</tbody>
</table>

3. Digest at 37°C for overnight in incubator,
   
   *(Note: Concentration of BAC DNA should be from 500 ng to 1 µg)*

2. Do Pulse-Field Gel Electrophoresis

3. Large Scale BAC Extraction

   **CsCl Method (Without Kit)**
   
   *(Note: As much as possible, do not vortex or mix vigorously to prevent breakage of BAC DNA)*

1. Culture 1 colony in 1 ml LB-chloramphenicol at 37°C for a minimum of 6 hrs.
2. Transfer culture to autoclaved 300 ml of LB Broth treated with Chloramphenicol.
3. Culture overnight at 37°C in incubator/shaker.
4. Transfer to autoclaved Nalgene bottles.
5. Centrifuge at 3,500 rpm for 5 min.
6. Decant supernatant.
7. Add Solution I (10 ml for every 300 ml bacterial medium) using 15 ml corning screw cap tube.
8. Vortex to resuspend pellet.
9. Add 10 ml each of freshly prepared Solution II (0.2 N NaOH and 2% SDS).
10. Shake gently for 15 seconds.
11. Stand on ice for 5 minutes.
12. Add 15 ml of Solution III.
13. Shake gently for about 1 min.
14. Stand on ice for about 5 min.
15. Centrifuge at 6,000 rpm for 25 min.
16. Transfer supernatant to clean Corning tube.
17. Add 3-5 ml equilibrated phenol to sample.
18. Shake vigorously for 1 min.
19. Centrifuge at 6,000 rpm for 20 min.
20. Pipette supernatant and transfer to another Corning tube.
21. Divide into two each sample.
22. Put equal volume of isopropanol.
23. Stand for 10 min.
24. Centrifuge for 6,000 rpm for 5 min.
25. Decant supernatant.
26. Wash with 30 ml of 75% ethanol.
27. Shake gently to rinse the sides.
28. Decant supernatant I
29. Dry using paper towels for about 30 min.
30. Put 2 ml of 1X TE in every sample.
31. Put 10 ul of RNase.
32. Incubate at 37°C for 1-2 hrs or at 4°C overnight.
33. Samples are now processed for ultracentrifugation.

4. Positive fragment ligation and culture

1. Cut concentrated BAC with the same restriction enzyme as Third screening.
2. Run in 1% agarose gel.
3. Cut out fragments, clean then purify.
4. Ligate in appropriate vector e.g. pHSG or pUC.
5. Transform in appropriate competent cells.
6. Spread in appropriate LB agar - antibiotic plate and culture overnight.
7. Restreak white colonies.

5. Colony hybridization

1. Prepare membrane similar to 2nd screening except the following:
   - Use the same probe employed in 1st and 2nd screening,
   - Since membrane is small, use the bottle not plastic bag,
- Use 50 ml volume for the hybridization buffer,  
- Use 100 ml volume for the wash buffer,  
- Do only Steps 1-8

**RNA interference (double-stranded RNA)**

**Materials**

RNA loading buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>: 50%</td>
</tr>
<tr>
<td>1mM EDTA</td>
<td>: 1 mM EDTA</td>
</tr>
<tr>
<td>bromophenol blue</td>
<td>: 0.4 bromophenol blue</td>
</tr>
<tr>
<td>ethidium bromide</td>
<td>: 1mg/ml</td>
</tr>
</tbody>
</table>

*dispense RNA loading buffer into aliquots and store at -20°C

RNA Sample buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0 ml deionized formamide</td>
<td></td>
</tr>
<tr>
<td>3.5 ml 37% formaldehyde</td>
<td></td>
</tr>
<tr>
<td>2.0 ml MOPS buffer</td>
<td></td>
</tr>
</tbody>
</table>

*dispense into aliquots and store at -20°C for up to six months

TE buffer

<p>| |</p>
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris HCl</td>
</tr>
<tr>
<td>1 mM EDTA</td>
</tr>
</tbody>
</table>

TE saturated (Phenol:Chloroform:iso-amyl alcohol)

Mix equal part of TE buffer and phenol and allow the phases to separate. Then mix 1 part of lower, phenol phase with 1 part of chloroform iso-amyl alcohol (24:1)
Molecular Biology Protocols for Aquatic Organisms

MOPS buffer

\[
\begin{align*}
0.2 \text{ M MOPS (pH 7.0)} \\
50 \text{ mM Sodium Acetate} \\
5 \text{ mM EDTA (pH 8.0)}
\end{align*}
\]

Stock Solutions

\[
\begin{align*}
1 \text{ M Tris} \\
0.5 \text{ M EDTA (pH 8)}
\end{align*}
\]

50x TAE Buffer

\[
\begin{align*}
242 \text{ grams Tris base} \\
57.1 \text{ mL glacial acetic acid} \\
100 \text{ mL 0.5 M EDTA, pH 8.0} \\
\text{Bring up to 1.0 L with distilled water}
\end{align*}
\]

Preparation of agarose gel for RNA

Chemicals needed in cleaning tanks:
- 1% SDS
- 3% \( \text{H}_2\text{O}_2 \)
- Absolute EtOH

Cleaning of electrophoresis tanks
1. Clean electrophoresis tanks by using 1% SDS.
2. Rinse with distilled water
3. Rinse with absolute EtOH
4. Soak in 3% \( \text{H}_2\text{O}_2 \) for 10 mins
5. Rinse tanks with DEPC-treated water before use.

Gel Preparation

1. Prepare 1.5% agarose gel containing 0.5 \( \mu \text{g/ml Ethidium Bromide} \).
2. Add 1-2 \( \mu \text{l} \) of RNA to 18-20 \( \mu \text{l} \) of RNA sample buffer.
3. Add 2-5 \( \mu \text{l} \) of RNA loading buffer.
4. Heat the sample for 5-10 mins at 65-70\(^\circ\)C prior to loading.

Citrate saturated phenol (pH 4.5): chloroform:iso-amyl(25:24:1)
Isopropanol
Ethanol (70 and 95%)
Methods

A. Pointers in preparing the template for transcription (using T7 polymerase)

- Design primers such that the target region of interest will not have significant homology with other genes (perform BLAST SEARCH using the region of interest)
- Incorporate T7 in both the forward and reverse primer, pair them both with gene specific primers.

![Primer design](image)

- Perform PCR separately using the most stringent condition possible.
- Make sure to work in an RNAse-free system from the preparation of the template up to the purification of dsRNA.

B. Production of dsRNA using T7 Ribomax Express Large Scale RNA Production System

Set-up the appropriate reactions at room temperature. The frozen RiboMAX T7 2X will contain precipitate that can be dissolved by warming the buffer at 37°C and mixing well.

I. Add reaction components in the order shown; be careful to dissolve the DNA template in water before adding it to the reaction.

<table>
<thead>
<tr>
<th>T7 Reaction Component</th>
<th>Sample Rxn</th>
<th>Control Rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>RiboMAX Express T7 2X Buffer</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>Linear DNA template (1 µg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM express + control</td>
<td>1 µl</td>
<td></td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>7 µl</td>
<td></td>
</tr>
<tr>
<td>Enzyme Mix T7 Express</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
2. Mix gently and incubate at 37°C for 30 min.

* frozen RiboMAX T7 2X will contain precipitate that can be dissolved by warming the buffer at 37°C and mixing well.

! do not freeze transcription reactions. After transcription reaction is complete, proceed directly to annealing.

Check integrity of transcripts in 1.5% agarose. Do not forget to add Et Br to RLB 0.5µg/ml 3 µl from 25mg/ml stock in 150 mL agarose gel.

II. Perform electrophoresis under standard condition used for the analysis of DNA samples

III. Annealing of dsRNA

1. To anneal RNA strands, mix equal volumes of complementary RNA reactions together and incubate at 70°C for 10 min.
2. Slowly cool at room temperature for about 20 min.

**check again in agarose gel following protocol #2

IV. Proceed to purification

**check again in agarose gel following protocol #2

V. Removing DNA template and Unincorporated rNTPs following transcription

1. Add RQ1 RNase-Free DNAse to a concentration of 1 unit per microgram of template DNA.
2. Incubate for 15 min at 37°C
3. Extract with 1 volume of citrate saturated PCI. Vortex for 1 min and spin in a microcentrifuge for 2 min.
4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of Chloroform:iso-amyl (24:1). Vortex for 1 min and centrifuged as described in step 3.
5. Transfer the upper, aqueous phase to a fresh tube. Any transferred chloroform can be removed by performing a quick spin (10 sec) in a microcentrifuge followed by removal of the bottom phase with a micropippete.
6. Add 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of iso-propanol or 2.5 vol of 95% EtOH. Mix and place on ice for 2-5 min. Spin at top speed in a microcentrifuge for 10 mins.

7. Carefully pour off or aspirate the supernatant and wash pellet with 1 ml of 70% EtOH.

8. Dry the pellet under vacuum and resuspend the RNA sample in TE or Nuclease free water to a volume identical to that of the transcription reaction.

9. Store at -70°C.

VI. **Check again in agarose gel** (do not forget to load the original transcript or single stranded transcript)

VI. **Quantification of RNA**

1. Dilute 2 µl of RNA in 68 µL of DEPC water. Read absorbance at 260 nm.

2. Alternatively, prepare a 1:100 to 1:300 dilution of the RNA and read absorbance at a wavelength of 260 nm.

*one 260 unit equals approximately 40µg/ml of RNA.

**In situ hybridization (RNA)**

**Materials:**

RNA friendly fixative (Hasson *et al.* 1997)

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% formalin</td>
<td>349 ml</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>407 ml</td>
</tr>
<tr>
<td>DEPC H2O</td>
<td>222 ml</td>
</tr>
<tr>
<td>Ammonium hydroxide</td>
<td>22 ml</td>
</tr>
<tr>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

MAS (Modified Alsever Solution) Anti-coagulant: (Patat *et al.* 2004)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>27 mM Sodium citrate</td>
<td></td>
</tr>
<tr>
<td>336 nM NaCl</td>
<td></td>
</tr>
<tr>
<td>115 mM glucose</td>
<td></td>
</tr>
<tr>
<td>9 mM EDTA (pH 7.0)</td>
<td></td>
</tr>
</tbody>
</table>
DEPC-treated water

| 0.1% (DW 1 liter/DEPC 1 ml) of diethyl pyrocarbonate in DW shake vigorously until all the DEPC globules have disappeared or keep in 37°C for 1 hr or over night
| Autoclave (to break down DEPC into CO2 and EtOH)

Proteinase K

| (5 μg/ml) 12.5 ul of stock proteanase K (20mg/ml) 50 ml PBS

4x SSC

| 0.6M NaCl, 0.06M Sodium citrate. (35.06 g NaCl, 17.65 Sodium citrate in 1 liter DEPC-treated water)

10x PBS

| PBS tablets supplied by a company

PBS glycine

| 2.0 mg/ml glycine in 1xPBS

Acetylation buffer

| 0.1M Triethanolamine pH 8.0
50% formamide in 2x SSC:

25 ml of formamide in 25 ml 4x SSC.

NTE:

0.5M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA. (29.22 g NaCl, 1.21 g Tris-HCl, 0.372 g EDTA)

RNase A

(20 ug/ml) 100 ul stock RNase A in 50 ml NTE. (RNase A stock is 10 mg/ml).

1% H$_2$O$_2$

30 ul of 30% H$_2$O$_2$ in 970 ul DEPC water.

Methods:

I. Sample preparation (shrimp hemolymph)

1. Withdraw hemolymph from shrimp using a DEPC prepared anti-coagulant.
2. Spin at 10,000 rpm for 10 min at 4 °C.
3. Remove supernatant and re-suspend hemocyte in RNA friendly fixative for 10 min on ice.
4. Wash 2x with PBS
5. Re-suspend to a final concentration of 10$^6$ cells/ml in PBS containing 50% ethanol (this can be stored at -20 °C for 3 months)
6. Before hybridization, drop the fixed cell suspension into slide coated with poly-L-lysine.
7. Air-dry at room temperature.
II. Probe preparation

Primer design

1. Design primers appropriate for the gene of interest.
2. Incorporate SP6 and T7 promoter to the anti-sense and sense strands respectively.
3. Amplify using the most stringent PCR condition.

Purification of PCR product

1. Purify by PCI purification method or if there are non-specific products, gel purify using EASY-TRAP (Takara).
2. Add equal volume of ddH$_2$O to the PCR product.
3. Add equal vol of PCI, spin at 12,000 rpm for 15 min
4. transfer supernatant (be careful not to get the organic phase)
5. Add 1 ml of 99% EtOH.
6. Add 10 µl of 3 mM Na Acetate.
7. Centrifuge at 12,000 rpm for 15 min.
8. Discard supernatant.
9. Dissolve pellet in 20 µl of ddh$_2$0, get 2 µl and run in gel.
10. Check purified products in agarose gel.
11. Quantify by spectrophotometry.

Probe labeling with DIG

In vitro transcription reaction

<table>
<thead>
<tr>
<th>Sense Strand</th>
<th>in µl</th>
<th>Anti-Sense Strand</th>
<th>in µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x transcription buffer</td>
<td>2</td>
<td>10x transcription buffer</td>
<td>2</td>
</tr>
<tr>
<td>*NTP labeling mixture</td>
<td>2</td>
<td>NTP labeling mixture</td>
<td>2</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1</td>
<td>RNase inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>T7 polymerase</td>
<td>2</td>
<td>SP6 polymerase</td>
<td>2</td>
</tr>
<tr>
<td>Purified DNA template (0.5-1.0µg)</td>
<td>-</td>
<td>Purified DNA template (0.5-1.0µg)</td>
<td>-</td>
</tr>
<tr>
<td>DEPC H$_2$O</td>
<td>-</td>
<td>DEPC H$_2$O</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

*NTP labeling mixture (each contains 10mM ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM DIG-UTP, pH 7.5)

1. Mix reagents and spin down briefly.
2. Incubate at 37$^0$C for 2 hrs.
3. Add 2 units of RNase free DNaseI (incubated at 37$^0$C for 2 hrs)
4. Add 1 µl of 0.5 µl of 0.5 M EDTA (pH 8.0) to stop the reaction.
5. Check labeled RNA probe in 1% agarose.

**Purification of labeled RNA transcript**

*a. Manual*

1. Add 500 µl 99% EtOH + 2.0 ul 3M NaOAc in DEPC.
2. Mix well (a precipitate will be formed, centrifuge at 12,000 rpm, 4 °C.
3. Air dry (but not completely)
4. Dissolve in 20 µl DEPC, keep in -20 °C.
5. Check RNA concentration by running 2 µl RNA probe on agarose gel.

**Hybridization**

*** Before the start of this experiment warm formamide and hybridization buffer (ISHR7) at 42°C until use in step 6.

All the steps below requires 2-3 liters of DEPC treated dH20 for preparing the solution and washing the slide containers, jars, (need at least 21 jars each jar contain 50.0 ml solution)

*a. Proteinase K treatment*

1. Dip slide in 1x PBS room temp. (RT) in 5 min. (To decrease background caused by endogenous peroxidase activity).
2. Dip slide in 0.3-1.0% H2O2 in 1x PBS RT in 10-60 min.
3. Wash in PBS RT in 5 min. (Warm PBS at 37°C before adding proteinase K at a concentration of 5 µg/ml)
4. Dip slide in proteanase K at 37°C in 10 min
5. Dip slide in PBS glycine RT in 10 min. (to remove prot).
6. Dip slide in PBS RT in 3 min.
7. Dip slide in PBS RT in 3 min.

*b. Acetylation* (through this step use the same jar) to decrease non specific background

1. Dip slide in 50 ml acetylation buffer RT.
2. Drop acetic anhydride (ISHR4) drop by drop of 250 µl within 5 min shaking.
3. Continue shaking RT for 15 min.shaking.
c. Washing

1. Dip slide in 1st 4x SSC at RT for 10 min.
2. Dip slide in 2nd 4x SSC at RT for 10 min.

d. Prehybridization

1. Dip slide in 50% formamide in 2x SSC at 42°C for 30 min.

e. Hybridization (DAKO pen may help to minimize the probe volume)

1. Mix two sense RNA probes in the same tube and two antisense RNA probes in the same tube,
2. Add 100 μl/tube warm hybridization buffer (ISHR7) continue warm at 42°C. Some recommend denature RNA probe at 75°C for 5 min before drop on tissue slide. (this step can be performed while prehybridization; hybridization buffer is quite sticky, cut pipette tip before take it).
3. Air dry slide but not completely.
4. Place the slide as shown in figure.
5. Drop the warm probes just for cover the slide (20-50 μl) and cover with paraffilm (it is better to use cover slide either oven baked at 200°C or straight from the box).
6. Place the slide in a sealed container with Whatman filter paper soaked in 2x SSC plus 50% formamide. It is important to include formamide otherwise the formamide in the hybridization buffer evaporates during incubation.
7. Incubate at 42°C for 16 hrs.
**f. Washing and RNase A treatment**

1. Dip slide in 1\textsuperscript{st} 50\% formamide in 2x SSC at 42°C for 20 min.
2. Dip slide in 2\textsuperscript{nd} 50\% formamide in 2x SSC at 42°C for 20 min.
3. Dip slide in 3\textsuperscript{rd} 50\% formamide in 2x SSC at 42°C for 20 min.
4. Dip slide in NTE at 37°C for 5 min. (Warm NTE at 37°C before adding RNase)
5. Dip slide in 20 μg/ml RNase A/NTE at 37°C for 30 min.
6. Dip slide in NTE at 37°C for 5 min.
7. Dip slide in 1\textsuperscript{st} 0.1x SSC at 42°C for 20 min.
8. Dip slide in 1\textsuperscript{st} 0.1x SSC at 42°C for 20 min.
9. Dip slide in 1\textsuperscript{st} 0.1x SSC at 42°C for 20 min.
10. Dip slide in 1\textsuperscript{st} 0.1x SSC at RT for 20 min and with shaking.
11. Dip slide in 1\textsuperscript{st} 0.1x SSC at RT for 20 min and with shaking.
12. Dip slide in 1\textsuperscript{st} 0.1x SSC at RT for 20 min and with shaking.
13. Air dry.

**g. Mounting**

Drop mount media on slide, then cover with cover slide and seal with nail polish.
Chapter II: Working with proteins

Commonly used reagents

A. 1.5 M Tris-HCl pH 8.8 (200 ml)

1. For 200 ml mix the following reagents:
2. Adjust to pH 8.8 with 5 N NaOH (optional).
3. Add deionized H₂O to a final volume of 200 ml.
4. Autoclave.
5. Place at room temperature for storage.

B. 1 M Tris-HCl pH 6.8 (200 ml)

1. For 200 ml mix the following reagents:
2. Adjust to pH 6.8 with 5 N NaOH (optional).
3. Add deionized H₂O to a final volume of 200 ml.
4. Autoclave.
5. Place at room temperature for storage.

C. 10X TBS (500 ml)

1. For 500 ml mix the following reagents:
   - Tris = 30.0 g
   - NaCl = 80.0 g
   - KCl = 2.0 g
   - dH₂O = raise to 1 L
2. Adjust to pH 7.5 with HCl.
3. Place at room temperature for storage.
D. 1X TBST (1 L)

1. For 1 Liter mix the following reagents:
   - 1X TBS  = 1 L
   - Tween 20  = 500 ul
2. Place at room temperature for storage.

E. Transfer Buffer (500 ml)

1. For 500 ml mix the following reagents:
   - Tris  = 6.05 g
   - Glycine  = 7.20 g
   - Methanol  = 100 ml
2. Add deionized H$_2$O to a final volume of 500 ml.
3. Place at room temperature for storage.

F. SDS Gel Electrophoresis Buffer

1. For 1 Liter mix the following reagents:
   - Tris  = 3.0 g
   - Glycine  = 14.4 g
   - 20% SDS  = 5.0 ml
2. Add deionized H$_2$O to a final volume of 1 Liter.
3. Place at room temperature for storage.

G. Coomasie Brilliant Blue Stain:

1. For 100 ml mix the following reagents:
   - Methanol  = 50.00 ml
   - dH$_2$O  = 40.00 ml
   - Brilliant Blue R  = 0.25 g
   - Glacial Acetic Acid  = 10.00 ml
2. Place at room temperature for storage
H. 2X SDS Sample Buffer

- 0.25 M Tris-HCl (pH 6.8) = 25 ml
- SDS = 2 g
- dH₂O = 9 ml
- Glycerol = 10 ml
- BPB (?) = 5 mg

Bacterial expression vectors (pET 32, pQE, pNCMO2)
Materials

Drug Free LB Broth

- Tryptone = 1.0 g
- Yeast = 0.5 g
- NaCl = 0.5 g

Scale up to 100 ml using dH₂O.
Adjust to pH 7 with 5 N NaOH (optional).
Add distilled H₂O to a final volume of 100 ml.
Autoclave.

2 xYT

- Tryptone = 1.6 g
- Yeast = 1.0 g
- NaCl = 0.5 g

Scale up to 100 ml using dH₂O.
Adjust to pH 7 with 5 N NaOH (optional).
Add distilled H₂O to a final volume of 100 ml.
Autoclave.
LB Agar

- Tryptone = 1.0 g
- Yeast = 0.5 g
- NaCl = 0.5 g
- Agar = 1.5 g

Scale up to 100 ml using dH2O.
Adjust to pH 7 with 5 N NaOH (optional).
Add deionized H2O to a final volume of 100 ml.
Autoclave.
Put in 50°C incubator for storage.
Dispense in Petri dish aseptically.

LB-antibiotics

Mix the following reagents (for 100 ml): (note: cool down Drug Free LB Broth to about 50°C before adding antibiotic to prevent the latter degradation due to high temperature)

- Drug Free LB Broth = 100 ml
- 10 mg/ml antibiotic = 100 μl

Mix gently.

LB-antibiotics/X-gal/IPTG agar plates for Blue/White selection

Mix the following reagents (for 100 ml): (note: cool down LB Agar to about 50°C before adding antibiotic to prevent the latter degradation due to high temperature)

- LB Agar = 100 ml
- 10 mg/ml Kanamycin = 100 μl
- X-gal = 50 μl
- IPTG = 20 μl

Mix gently then pour to Petri dishes, about 15-20 ml/dish.
Swirl gently to spread out the broth mixture in the dish.
Cool and dry for 5-15 min. at room temperature (note: Petri dishes should be turned upside down).
Wrap with Saran wrap then store at 4°C.
1% Agarose gel

| - Agarose | = 1 g |
| - 1X TBE  | = 100 ml |

Dissolve/ boil in microwave for about 2 min.
Add 1 µl of ethidium bromide.
Swirl gently.

Solutions I, IIA, IIB and III

1. Mix the following reagents

1.1. Solution I (for 200 ml)

| - Glucose | = 1.8 g |
| - 1 M Tris-HCl | = 5.0 ml |
| - EDTA | = 0.74 g |
| - dH₂O | = 100 ml |

1.2. Solution IIA (for 100 ml)

| - NaOH | = 1.6 g |
| - dH₂O | = 100 ml |

1.3. Solution IIB (for 100 ml)

| - SDS | = 2.0 g |
| - dH₂O | = 100 ml |

1.4. Solution III (for 500 ml)

| - KOaC | = 147 g |
| - glCH₃COOH | = 57.5 ml |
| - dH₂O | = 500 ml |
I. Methods

Preparation of vector-insert construct

Extraction of Insert from T-Vector Easy using restriction enzyme

1. Mix the following in 0.6 ml Eppendorf tube:

- T-Vector Easy with G-CSF Rhis6 insert = 5.0 µl (1 ug)
- 10 X H Buffer (for EcoR1) = 4.0 µl
- EcoR1 = 2.0 µl
- dH20 = 4.0 µl

Total volume of reaction = 15.0 µl

2. Incubate for 4 hrs at 37°C.
3. Run in 2% agarose gel to check insert restriction and for purification.

Purification of extracted insert

4. Cut agarose gel containing DNA as shown by U.V. light and place in 1.5 ml microfuge tube labeled accordingly,
5. Add 3X volume of NaI and 1/2 volume of Melting Buffer (Toyobo Purification Kit), e.g. in 100 µg of gel put 300 µl NaI and 50 µl of Melting Buffer.
6. Incubate for 10 min at 50°C.
7. Add 5 µl silica milk (Toyobo Purification Kit) to mixture.
8. Mix well by vortexing or finger flicking and incubate for 5 min on ice.
9. Spin down for about 5 sec at 9,000 rpm and then pipette out supernatant (leave pellet).
10. Perform the following washing for 3X: (note: leave a small amount of supernatant on the last washing step then pipette out)
11. Dissolve in 5 µl of distilled H2O.
12. Incubate for 3 min at 45°C – 55°C using hot H2O bath and thermometer.
13. Spin down for 2 min at 12,500 rpm.
14. Transfer supernatant to clean microfuge tube.

Restriction of vector with restriction enzyme

1. Mix the following reagents:

- pBK-CMV plasmid (1 µg/µl) = 1.0 µl
- 10 X H Buffer (for EcoR1) = 2.0 µl
- EcoR1 = 0.5 µl
- dH20 = 6.5 µl

Total volume of reaction = 10.0 µl

2. Incubate at 37°C for 2 hrs.
3. Run in 1% agarose gel.

**Purification of restricted vector**

1. Add 35 µl of PEG to DNA sample, mix well then spin down.
2. Stand on ice for 30 min.
3. Centrifuge at 13,000 rpm at 4°C for 10 min.
4. Aspirate supernatant using micropipette.
5. Add 500 µl of 75% Ethanol.
6. Add 1 µl of 10 mg/ml glycogen and 3.3 µl of 3M NaOAC.
7. Centrifuge at 13,000 rpm at 4°C for 5 min.
8. Discard supernatant by aspiration.
9. Centrifuge again in order to discard absolutely supernatant.
10. Dry up tubes at room temperature (*note: tubes are turned upside down on Kimwipes*).
11. Add 50 µl of distilled H2O or TE Buffer.
12. Store at -20°C for subsequent use.

**Depshorphorylation of digested Vector with Calf Intestinal Alkaline Phosphatase (CIAP)**

1. Add 5 µl of CIAP Buffer and 2 µl of CIAP to 50 µl purified pBK-CMV plasmid,
2. Incubate at 37°C for 1 hr.
3. If too small, scale up the mixture with dH2O.

**Purification of dephosphorylated vector**

1. Add equal volume of Phenol-Chloroform to plasmid mixture.
2. Mix well/ shake vigorously for about 30 sec.
3. Centrifuge for at least 12,500 rpm for 20 min in a refrigerated centrifuge.
4. Transfer supernatant to a clean Eppendorf tube using pipette (*note: avoid touching the middle portion*).
4. Add 500 μl of 99% Ethanol.
5. Add 1 μl of glycogen and 3.3 μl of 3M NaOAC.
7. Stand for about 10 min at room temperature.
8. Centrifuge for 10 min at 11,000 rpm using a refrigerated centrifuge.
9. Decant supernatant then dry in room temperature using Kimwipes.
10. Resuspend pellet in 50 μl dH₂O or 1X TE Buffer.

**Ligation of insert to Vector**

**Option A**

1. Resuspend in a volume of 5mM Tris-HCl (pH 7.5) and 0.1 mM EDTA.
2. Mix the following reagents:

   - Vector (0.1 μg/μl) = 1.0 μl
   - G-CSF Rhas6 insert (0.07 pmol ends/μl) = 2.0 μl (5 μl)
   - 10mM rATP (pH 7.0) = 1.0 μl
   - 10x Ligase Buffer = 1.0 μl
   - ddH₂O = 4.5 μl
   - T4 DNA ligase (4 Weiss U/μl) = 0.5 μl

   Total volume of reaction = 10.0 μl

3. Ligate for overnight at 4°C. (When using blunt ends, ligate overnight at 12-14°C).

**Option B**

1. Mix the following reagents:

   - Vector (0.1 μg/μl) = 1.0 μl
   - Insert (0.07 pmol ends/μl) = 2.0 μl (5 μl)
   - Ligation High (TOYOBO) = 3.0 μl

   Total volume of reaction = 6.0 μl

2. Ligate at 16°C for 30 min. or overnight at 4°C. (When using blunt ends, ligate overnight at 12-14°C).
Transformation of E.coli cells with Vector/insert

11. Mix 5 µl of vector/insert to 50 µl competent *E. coli* cells in a microfuge tube labeled appropriately.
12. Stand on ice for 30 min.
13. Heat shock cells at 42°C for 45 sec to 1 min then stand on ice for 2 min.
15. Cover microfuge tubes with parafilm.
16. Put in H₂O bath for 1 hr with shaking at 37°C.
17. Centrifuge mixture for about 2 min at 6,000 rpm.
18. Remove half of the supernatant to concentrate mixture then dissolve pellet using pipette.
20. Incubate for overnight at 37°C.
21. Check for the presence of bacterial colonies the next day.

Culture of transformed XL1-Blue MRF* E. coli

1. Select 3-5 colonies and streak in new LB-antibiotic/X-gal/IPTG agar plates.
2. Incubate for overnight at 37°C.
3. Transfer 1-2 white colonies to test tubes containing 2 ml of LB-Kanamycin (use sterile conditions).
4. Place culture mixture to shaker-incubator for overnight at 37°C.

Insert check

Colony PCR

1. Amplify G-CSF cDNA using M13 Forward and RHis6 primers and check for base pair content (use this as positive control).
2. Take a small colony and suspend it in 5 µl of H₂O in a PCR tube.
3. Heat for 5 min at 95°C and then spin the condensation down in a microfuge.
4. Do regular PCR reaction.
5. Run in 1% Agarose gel.

Restriction

1. Mix the following reagents:

   - EcoR1 = 0.5 µl
- 10x H Buffer = 2.0 µl
- dH20 = 5.5 µl
- pBK-CMV plasmid = 2.0 µl

Total volume of reaction =10.0 µl

2. After mixing, dispense to each sample tube containing 2 µl of plasmid,
3. Spin down for 5 sec at 120 rpm.
4. Incubate for 2 hrs at 37°C.
5. Run in 1% agarose gel to check insert.

**Sequencing**

1. Sequence the purified plasmid using appropriate vector primers.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

**Materials**

- Distilled/deionized water
- Acrylamide (LR)
- 1.5 M Tris HCL, pH 8.8
- 1 M Tris HCl, pH 6.8
- 10% SDS
- 10% APS
- TEMED
- Marker (Precision Plus protein etc.)

Coomassie Blue Stain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>- 50 ml</td>
</tr>
<tr>
<td>D H2O</td>
<td>- 40 ml</td>
</tr>
<tr>
<td>Brilliant Blue R</td>
<td>- 0.25 g</td>
</tr>
<tr>
<td>Glacial Acetic</td>
<td>- 10 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>-100 ml</strong></td>
</tr>
</tbody>
</table>
2X SDS Sample Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Tris-HCl, pH 6.8</td>
<td></td>
<td>25 ml</td>
</tr>
<tr>
<td>SDS</td>
<td></td>
<td>2 g</td>
</tr>
<tr>
<td>D H₂O</td>
<td></td>
<td>9 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td>10 ml</td>
</tr>
<tr>
<td>Bromphenol Blue</td>
<td></td>
<td>5 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>---------</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50 ml</td>
</tr>
</tbody>
</table>

*Before use: add 100 μl/ml of β-mercaptoethanol*

**Methods**

1. **Plate preparation**


2. Place rubber seal into plates, secure with green clips.

3. Select the appropriate comb for the gel wells.
4. Mix the following accordingly into a 50 ml corning tube:

<table>
<thead>
<tr>
<th>Reagents (For 2 plates)</th>
<th>12%</th>
<th>15%</th>
<th>21%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (LR) 50%</td>
<td>3.84 ml</td>
<td>0.67 ml</td>
<td>4.8 ml</td>
</tr>
<tr>
<td>1.5 M Tris HCl, pH 8.8</td>
<td>4 ml</td>
<td>---</td>
<td>4 ml</td>
</tr>
<tr>
<td>1 M Tris HCl, pH 6.8</td>
<td>---</td>
<td>0.75 ml</td>
<td>---</td>
</tr>
<tr>
<td>10% SDS</td>
<td>160 μl</td>
<td>60 μl</td>
<td>160 μl</td>
</tr>
<tr>
<td>10% APS (new)</td>
<td>54 μl</td>
<td>20.4 μl</td>
<td>54 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μl</td>
<td>6 μl</td>
<td>8 μl</td>
</tr>
<tr>
<td>D H$_2$O</td>
<td>7.97 ml</td>
<td>4.49 ml</td>
<td>7.01 ml</td>
</tr>
</tbody>
</table>

5. Mix first the running gel and load into the assembled plates as quickly as possible. Leave around 5 cm space for the stacking gel.

6. Load d H$_2$O and fill up to the rim.

7. Allow this gel to polymerize for about 30 min or until a separation phase is seen between the d H$_2$O and the gel. You could also use the remaining gel as a marker for polymerization.

8. Remove d H$_2$O using vacuum pump. Rinse the gel again by d H$_2$O and dry by aspiration and/or by kimwipes.
9. Mix the stacking gel and load in the assembled plates as quickly as possible. Load carefully to avoid creating bubbles.

10. Place the comb into the plates (as shown below), then allow to polymerize for about 30 min. You could also use the remaining gel as a marker for polymerization.

![Complete SDS-PAGE set-up](image)

2. **Running samples**

1. Put appropriate amount of 2X SDS Loading Buffer to samples *e.g.*, 5 µl sample: 15 µl buffer.
2. Boil at 95°C for 15 to 30 minutes until the mixture changes color from dark red to bluish green color.
3. Load around 20 to 30 µl of sample to each well of the SDS-PAGE gel.
4. Do not forget to load marker *e.g.* Precision Plus Protein Standards or Kaleidoscope Prestained Standards by BIO-RAD.
5. Run at 90 min at 30 mV.
6. Perform visualization by Coomasie blue staining or Western Blotting.

3. **Coomasie blue staining**

1. Transfer gel into a container *e.g.* square plates.
2. Overlay with freshly prepared Coomasie blue stain about 25 ml.
3. Do mild shaking from 30 minutes to overnight.
4. After shaking, pour off the staining solution (it can be stored because it is reusable).
5. Wash gel with dH₂O thrice.
6. Put sufficient amount of dH₂O then boil in microwave for about 10 min.
7. Discard then soak in new dH₂O. Repeat No.6 until staining solution is removed from the gel.
8. Visualize using Gel Lamp Reader.
Western blotting

Materials

Methanol
Membrane (ATTO clear blot)
- cut according to size of gel
3M Whatman paper
- cut according to size of gel
Bovine Serum Albumin (BSA)
Tris
Glycine
SDS 20%
Methanol or Ethanol
BCIP/NBT

10X TBS

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>30.3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>3.8 g</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>1.8 g</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Raise to</td>
<td>1 L with dH2O</td>
</tr>
<tr>
<td>Adjust to pH</td>
<td>7.5 then sterilize.</td>
</tr>
</tbody>
</table>

1X TBST

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X TBS</td>
<td>100 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>900 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>500 μl</td>
</tr>
</tbody>
</table>

Sterilize.
### Blocking buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TBST</td>
<td>20 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>0.4 g</td>
</tr>
</tbody>
</table>

Mix properly until homogenized.

### BCIP/NBT substrate

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCIP/NBT tablet</td>
<td>1 tablet</td>
</tr>
<tr>
<td>dH₂O</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Mix properly until homogenized.

### Transfer buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6.05 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.2 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

----------

Raise to 500 ml dH₂O

Mix with magnetic stirrer.

### Electrophoresis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

----------

Raise to 1 L dH₂O

Mix with magnetic stirrer.
Methods

Gel fractionation and membrane transfer

1. Fractionate proteins on SDS polyacrylamide gel.
2. Transfer the separated proteins from the gel to a membrane (ATTO clear blot):
   - soak membrane in methanol for 20 sec
   - sandwich membrane in 10 layers of 3M paper then soak in transfer buffer
   - put 5 layers of 3M paper on transfer apparatus (ATTO)
   - put membrane on 3M paper
   - put gel on membrane
   - put 5 layers of 3M paper on gel
   - add ~ 10 ml of transfer buffer
   - put cover of transfer apparatus
   - connect with electrode 100-110 mA for 45 min – 1 hr

![Diagram of gel, membrane, and 3M paper]

Blocking the membrane

1. Transfer the blotted membrane to Petri dish and wash 3X with TBST.
2. Add 50 ml of blocking buffer and incubate for 1 hr at room temperature with gentle shaking.
3. Discard the buffer and wash 3 times (5 min each) with TBST.

1st Antibody binding (e.g. Anti-Xpress or Anti-His)

1. Prepare 1:5000 dilution of anti-His Ab (~ 1 µl of Anti-His Ab/ 5 ml of blocking buffer
2. Pour Ab/buffer mixture to the membrane in Petri dish.
3. Incubate for 1 hr at room temperature with gentle shaking.
4. Discard the Ab/buffer mixture. Alternatively, this can be stored at 4°C for subsequent use.
5. Wash the membrane with 20-30 ml TBST for 5 min at room temperature with gentle shaking (3X).

2\textsuperscript{nd} Antibody (\textit{e.g.} Anti-Mouse IgG)

1. Prepare the 2\textsuperscript{nd} Ab (Anti-Mouse IgG) by diluting 1 µl of 2\textsuperscript{nd} Ab with 10 ml of Blocking buffer (1:10,000).
2. Pour 2\textsuperscript{nd} Ab/buffer mixture to membrane in petri dish.
3. Incubate for 1 hr.
4. Wash the membrane with 20-30 ml TBST for 5 min at room temperature with gentle shaking (3X).

Visualization of reaction

1. Pour the BCIP/NBT substrate to the membrane in Petri dish.
2. Incubate in dark place for 2-5 min or up to 2 hrs.
3. Wash with dH2O to stop reaction.

Protein detection and purification

A. Small scale

Materials

- 1X PBS
- 1M IPTG
- SDS Buffer

Methods

1. Culture 1 colony of BL21+ clones with possible pET32 ligated vectors in 2 ml 2YT broth + Chloramphenicol/ Ampicillin overnight at 37°C.
2. Centrifuge culture at 5,000 rpm for 10 minutes.
3. Aspirate supernatant using vacuum pump.
4. Resuspend pellet with 300 µl of 1X PBS.
5. Take 50 µl of the mixture and combine with 50 µl of SDS buffer solution.
6. Boil for at least 10 min. then run in SDS-PAGE.
A. Large scale

Materials

Denaturing buffer (DB)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>6 g</td>
</tr>
<tr>
<td>Urea</td>
<td>480.5 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 ml</td>
</tr>
<tr>
<td>Raised to</td>
<td>100 ml d H₂O</td>
</tr>
</tbody>
</table>

Elution buffer (Imidazole)

- 500 mM Imidazole (1.7 g imidazole + 10 ml DB)
- 300 mM Imidazole (3 ml of 500 mM imidazole + 5 ml DB)
- 100 mM Imidazole (1 ml of 500 mM imidazole + 5 ml DB)
- 10 mM Imidazole (1 ml of 500 mM imidazole + 50 ml DB)

20% Ethanol

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>99% pure Ethanol</td>
<td>20 ml</td>
</tr>
<tr>
<td>d H₂O</td>
<td>80 ml</td>
</tr>
<tr>
<td>Total</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

1 M IPTG

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>2.38 g</td>
</tr>
<tr>
<td>d H₂O</td>
<td>10 ml</td>
</tr>
<tr>
<td>Total</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
Ionized agarose beads

1. Mix agarose beads gently
2. Pipette out 500 μl and put in microfuge tube.
3. Centrifuge at 6,000 rpm for 1 min at room temperature.
4. Wash with 1 ml of 20% ethanol (99% pure).
5. Centrifuge at 6,000 rpm for 1 min at room temperature.
6. Wash with 1 ml PBS.
7. Centrifuge at 6,000 rpm for 1 min at room temperature.
8. Wash with 1 ml Denaturing buffer.
9. Centrifuge at 6,000 rpm for 1 min at room temperature.

Dialysis bag

1. Cut dialysis bags in desired sizes.
2. Place in 500 ml beaker with d H₂O.
4. Boil for 10 to 13 min.
5. Store at 4°C until use.

10X PBS

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>11.5 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2 g</td>
</tr>
</tbody>
</table>

---------

Raised to - 1 L
Autoclave.

3 L of 1X PBS

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>300 ml of 10X PBS</td>
<td>- raised to 3 L</td>
</tr>
<tr>
<td></td>
<td>Autoclave.</td>
</tr>
</tbody>
</table>
Methods

1. Culture positive BL21+ clones in 3 ml YT broth + Chloramphenicol/Ampicillin overnight at 37°C.
2. Put 200 µl of culture to 200 to 300 ml YT broth + Cp/Ap then culture for about 6 hrs at 37°C or until mixture become cloudy.
3. Put to 200-300 µl of 1 M IPTG to the mixture then culture again for 24 hrs at 37°C.
4. Transfer culture in 250 ml centrifuge bottles then spin down at 6,000 rpm for 5 min.
5. Resuspend and wash in 50 ml PBS 3 times. From 2nd wshing, 50 ml corning tubes will be used. The final volume after 3rd washing shoud be 30 ml. Separate 50 µl of the washed culture for SDS-PAGE later.
6. Perform freeze-thaw method using -80°C freezer and 42°C water bath. Do this 2 to 3 times.
7. Divide the culture into 3 (about 10 ml in each corning tube). Dilute the mixtures to 50 ml final volume.
8. Perform sonication as follows:
   8.1. Select the big rod of the sonicating machine. This is used for the 50 ml corning tube while the small rod is for the microfuge tube.
   8.2. Turn the machine on, power switch located in the bottom.
   8.3. Select 20 sec as length sonication.
   8.4. Turn-off pulse.
   8.5. Set amplitude at 20 – 25.
   8.6. Wash rod with alcohol in between samples.
   8.7. Sonicate for 15X or more until mixture becomes translucent.
9. Spin down sonicated samples at 5,000 rpm for 10 min at 4°C.
10. Resuspend and wash with 1ml PBS once.
11. Spindown sonicated samples at 5,000 rpm for 10 min at 4°C.
12. Resuspend in 500 µl PBS. Separate 20 µl sample for SDS-PAGE later.
13. Take 200 µl of the resuspended mixture and dilute to 40 ml DB in 50 ml corning tube.
14. Shake for about 5 min.
15. Filter with a 0.45 μM Millipore using a 10 ml syringe.
16. Mix filtrate with 500 μl of ionized agarose beads.
17. Shake gently for about 5 min.
18. Pass to 5 ml polypropylene column accordingly:
   18.1. put mixture first
   18.2. put (wash) with 3 ml DB for 5 times
   18.3. put (wash) with 3 ml 10 mM imidazole
   18.4. ELUTE with 4 – 6 ml of 500 mM imidazole. Use 15 ml corning tube.
19. Dialyze protein as follows:

19.1. Close dialysis bags on one end using clips (orange or green). Fold 3X before clipping.
19.2. Load the eluted protein to the dialysis bag using a pipette.
19.3. Close the other end of the bag also using clips. Fold 3X before clipping.
19.4. Put in autoclaved 3 L 1X PBS and dialyze for 24 hrs or more.
19.5. After dialysis, transfer liquid inside the bag to 15 ml corning tube.

20. Store proteins at -80°C freezer.
21. Run protein samples in SDS PAGE.
Chapter III: Working with cells and tissues

Preparation of bacterial competent cells

A. For electroporation

Materials

dH₂O

Methods

*Note: All procedures should be done on ice and near the flame.*

1. Culture 1 colony of bacteria (DH5α, JM109, BL21+ etc) in 2 ml drug free 2YT broth overnight at 37°C with shaking.
2. Transfer the culture to 1 L drug free 2YT broth and incubate for 6 to 7 hrs at 37°C.
3. Centrifuge at 3,000 rpm for 3 min at 4°C.
4. Wash with distilled/deionized water.
5. Aliquot 50 µl of mixture to frozen microfuge tubes.
6. Store at -80°C.

B. For heat-shock

Materials

1 M CaCl₂

<table>
<thead>
<tr>
<th>CaCl₂</th>
<th>- 5.55 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>MillQ H₂O</td>
<td>scale up to 50 ml</td>
</tr>
<tr>
<td>Total</td>
<td>- 50 ml</td>
</tr>
</tbody>
</table>

100 mM CaCl₂

<table>
<thead>
<tr>
<th>1 M CaCl₂</th>
<th>- 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MillQ H₂O</td>
<td>scale up to 90 ml</td>
</tr>
<tr>
<td>Total</td>
<td>-100 ml</td>
</tr>
</tbody>
</table>
100 mM CaCl$_2$ with 10% Glycerol

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M CaCl$_2$</td>
<td>10 ml</td>
</tr>
<tr>
<td>60% glycerol</td>
<td>25 ml</td>
</tr>
<tr>
<td>MillQ H$_2$O scale up to 100 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100 ml</strong></td>
</tr>
</tbody>
</table>

Microfuge tubes in rack frozen at -80°C freezer.

**Method 1**

*Note: All procedures should be done on ice and near the flame.*

1. Culture 1 colony of bacteria (DH5α, JM109, BL21+ etc) in 2 ml drug free 2YT broth overnight at 37°C with shaking.
2. Transfer 200 ml of the culture to L-type or regular test tube with 10 ml drug free 2YT broth and stand for 2 hrs.
3. Place tubes on ice for about 10 min.
4. Put 1 ml each of the culture to the microfuge tubes (total 10 tubes). Do this near the flame!
5. Spin at 3,000 rpm for 3 min at 4°C ultracentrifuge.
6. Aspirate the supernatant using a vacuum pump. Do this as quickly as possible.
7. Add 200 μl of fresh and cold 100 mM CaCl$_2$ to the tubes with pellet.
9. Mix tubes 1 and 2, 2 and 3, and so forth.
10. Add 1 ml of 100 mM CaCl$_2$ to the remaining tubes.
11. Stand on ice for 30 min. with continuous shaking (to prevent bacteria from settling down).
12. Spin at 3,000 rpm for 3 min at 4°C ultracentrifuge.
13. Pipette out supernatant.
14. Put 330 μl of 100 mM CaCl$_2$ with 10% glycerol. Do this near the flame!
15. Mix tubes 1 and 2, 3 and 4, and so forth.
16. Aliquot 50 μl of mixture to new microfuge tubes.
17. Store at -80°C.

**Method 2**

1. Culture a colony of bacteria or 100 μl of competent bacterial cells into 10 ml drug free YT medium for 6 hours
2. Place tubes on ice for about 30 min.
3. Transfer culture to 15 ml corning tube.
4. Spin at 3,000 rpm for 3 min at 4°C ultracentrifuge.
5. Discard supernatant.
6. Resuspend pellet with 10 ml of 100 mM CaCl$_2$ by pipetting.
7. Put on ice for about 30 min.
8. Spin at 3,000 rpm for 3 min at 4°C ultracentrifuge.
10. Resuspend pellet in 3 ml of 100 mM CaCl$_2$ with 10% glycerol.
11. Aliquot in previously frozen Eppendorf tubes (-80°C) at 100 μl each.
12. Store at -80°C.

**Cell culture**

**Cells used**

HINAE (Hirame Natural Embryo Cell line) - FBS medium
YO-K - FBS medium

**Materials**

1X PBS

<p>| PBS tablet | - 1 pc |</p>
<table>
<thead>
<tr>
<th>D H$_2$O</th>
<th>- 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>- 100 ml</td>
</tr>
</tbody>
</table>
| **Autoclave.**

Fetal Bovine Serum (FBS)

2. Cool down.
3. Aliquot 50 ml of FBS to new 50 ml flasks.
4. Seal flasks with parafilm.

Antibiotic (Penicillin/Ampicillin) – (Commercial)
Trypsin solution (commercial)
Medium (commercial)
<table>
<thead>
<tr>
<th>Concentration</th>
<th>Medium</th>
<th>FBS</th>
<th>Penicillin/ Ampicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium + 20% + P/S</td>
<td>200 ml</td>
<td>50 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Medium + 15% + P/S</td>
<td>212.5 ml</td>
<td>37.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Medium + 10% + P/S</td>
<td>225 ml</td>
<td>25 ml</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

**Methods**

**1. Passage**

1. Discard old medium.
2. Wash each flask 25 cm² with 3-4 ml PBS. Shake/swirl gently for a few seconds.
3. Discard the PBS leaving a small amount.
4. Add about 2 ml trypsin solution to each flask using Pasteur pipette.
5. Swirl and keep cell line in trypsin for about 30 sec to 1 min. then pipette out half of the supernatant.
6. Tap flask using hands to detach the cells and dissociate them.
7. Add about 2 ml of medium (L-15 + 15% FBS + P/S). Pipette in and out the mixture to completely separate the individual cells.
8. Aliquot the mixture into 3 to 4 25 cm² flasks.
9. Put about 4 ml of medium (L-15 + 15% FBS + P/S) to each flask.
10. Incubate cells at 20°C for growth.

**Isolation of white blood cells from whole blood, kidney or spleen of Japanese flounder, *Paralichthys olivaceus***

**Materials**

1. Heparin dissolved in 1X PBS – 1000 units
2. Percoll solution
   - Mix the following in 50 ml corning tube:
     *note: precise measurement is needed*
   - 26.5 ml of Percoll
   - 2.95 ml 10X PBS
   - Adjust to 50 ml with 1X PBS
3. Mesh net – sterile
4. Percoll tubes
5. RPMI
6. 1X PBS (degassed)
**Methods**

**A. Whole blood collection**

1. Wash/flush syringe with heparin (1000 units),
2. Collect blood from the fish caudal artery and put in 50 ml corning tube,
3. Mix heparinized blood with RPMI to the ratio of 1:3. For example 10 ml of blood to 30 ml of RPMI,
4. Put on ice until use.

**B. White blood cell from kidney or spleen**

1. Dissect out kidney or spleen and put in 20 ml RPMI,
2. Slowly mash/filter tissue using a sterile mesh net and a syringe plunger on a 1X PBS
3. Put filtered mixture in 30 ml RPMI.
4. Put on ice until use.

**C. Cell separation**

1. Put 3.0 ml Percoll solution (Pharmacia, USA) into Percoll tube.
2. Add slowly 5.0 ml of blood-RPMI mix (*note: first 1.0 ml should be added very slowly to generate a sharp interface of blood and Percoll solution*).
3. Centrifuge 400x g at 4°C for 30 min using a swing rotor.
4. Pipette carefully the interface (middle layer) to a new 50 ml corning tube. (*note: leukocytes appear as a cloudy layer*).
5. Raise to equal volume or to about 30 ml using RPMI.
6. Put 3.0 ml Percoll solution (Pharmacia, USA) into Percoll tube.
7. Add slowly 5.0 ml of white blood cell-RPMI mix (*note: first 1.0 ml should be added very slowly to generate a sharp interface of blood and Percoll solution*).
8. Centrifuge 400x g at 4°C for 30 min using a swing rotor.
9. Pipette carefully the interface (middle layer) to a new 50 ml corning tube. *(note: leukocytes appear as a cloudy layer).*

10. Raise to equal volume or to about 30 ml using RPMI.

11. Centrifuge 400x g at 4°C for 10 min.

12. Discard supernatant carefully.

13. Wash with 5 ml 1X PBS (pH 7.2).

14. Centrifuge 400x g at 4°C for 10 min.

15. Remove supernatant carefully.

16. Resuspend in 1 ml of 1X PBS.

D. Primary culture

1. Place PBLs or head kidney and keep in 10 ml cold medium A. *(note: this should be done in the laminar flow hood with sterile reagents).*

2. For head kidney, slowly mash/filter tissue using a sterile mesh net and a syringe plunger on a 1X PBS.

3. Isolate cell suspension as same as from step 3 of cell separation.

4. Resuspend in medium B.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Medium A (ml)</th>
<th>Medium B (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X 1</td>
<td>X 4</td>
</tr>
<tr>
<td>Penicillin/streptomycin</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>RPMI</td>
<td>8.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Cell staining**

**Materials**

Giemsa stain (Commercial)
May-Grunwald-Giemsa (Commercial)
Glass slides (APS coated)
Cover slip
Methanol

Sorenson’s buffer, pH 7.4

Mix the following

KH$_2$PO$_4$ – 6.63 g
Na$_2$HPO$_4$ – 2.56 g
Raised to 1 L of distilled H$_2$O, adjust to pH 7.4.
Methods

1. Blood smears

1. Put 1 drop of blood in an APS coated slide and smear using another slide,

   ![Blood smear diagram]

2. Soak in methanol for a few seconds or in 10% formalin in 1X PBS for overnight.
3. Air dry for 1 to 2 hrs.
4. For Giemsa, soak in staining solution containing 150 ml Sorenson’s buffer and 1 ml Giemsa for 45 min.
5. For May-Grunwald-Giemsa, soak in staining solution containing 250 μl of stain and 100 ml methanol for 3 min. Wash in distilled H₂O for 1 min. Soak in 10% Giemsa (10 ml stain to 90 ml Sorenson’s buffer). Wash 2X in distilled H₂O for 5 min.
6. Air dry for 1 to 2 hrs.
7. Mount slides using cover slip and nail polish.

2. Tissues

1. Dissect out tissue from fish such as head kidney, spleen, thymus etc.
2. Slice the tissues with a sharp blade and blot in slide.

   ![Tissue slice diagram]

3. Soak in methanol for a few seconds or in 10% formalin in 1X PBS for overnight.
4. Air dry for 1 to 2 hrs.
5. For Giemsa, soak in staining solution containing 150 ml Sorenson’s buffer and 1 ml Giemsa for 45 min.
6. For May-Grunwald-Giemsa, soak in staining solution containing 250 μl of stain and 100 ml methanol for 3 min. Wash in distilled H₂O for 1 min.
Soak in 10% Giemsa (10 ml stain to 90 ml Sorenson’s buffer). Wash 2X in distilled H₂O for 5 min.

7. Air dry for 1 to 2 hrs.
8. Mount slides using cover slip and nail polish.

**Immunohistochemistry (Paraffin embedded tissues)**

**Materials**

- APS-coated cells
- Microtome
- Water bath
- 100% Ethanol
- 70 % Ethanol (70 ml EtOH in 30 ml DW)
- 50 % Ethanol (50 ml EtOH in 50 ml DW)
- 30 % Ethanol (30 ml EtOH in 70 ml DW)
- 1X PBS
- Xylene (used is ok)
- 0.3% Hydrogen Peroxide (H₂O₂) in Methanol

<table>
<thead>
<tr>
<th>2 ml H₂O₂</th>
<th>2 ml Methanol</th>
<th>198 ml</th>
</tr>
</thead>
</table>

- 5% Bovine Serum Albumin (BSA) in 1X PBS

<table>
<thead>
<tr>
<th>BSA</th>
<th>5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

- Blocking buffer

<table>
<thead>
<tr>
<th>BSA</th>
<th>0.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBST</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Methods**

**A. Slide preparation (paraffin section)**

1. APS coated superfrost slides should be used.
2. Parafin embedded samples are stored in 4°C for better cutting.
3. Room should be cold while cutting.
4. Prepare water bath by heating at 31°C.
5. Clean the microtome, then put oil on 2 sides.
6. Check blade is still sharp (*note: if not sharp, change with new blade in the drawer*).
7. Put, lift lever.
8. Determine thickness by adjusting screw to 2.5 to 3 um. First blade for adjusting, second blade for cutting.
9. When cutting, take sample by brush. Breathe into sample to prevent static and for Moisture.
10. Put cut sample in the 37°C water bath for about 5 seconds.
11. Air dry for 1 to 2 hrs.
12. Remove paraffin by heating at 65°C for 30 min.
13. Slides could be stored for a few months at –80°C.

B. Slide preparation (fresh smear)

1. Use APS coated slides.
2. Smear or blot tissues in the slide.
3. Air dry for 1 to 2 hrs.
4. Fix in 10% formalin in PBS for overnight.
5. Air dry for 1 to 2 hrs.
6. Slides could be stored for a few months at –80°C.

C. Washing

1. Put slides in xylene using coupling jar for 5 min 3X.
3. Wash with 100 % Ethanol for 5 min 1X.
4. Wash with 70 % Ethanol for 5 min 1X.
5. Wash with 50 % Ethanol for 5 min 1X.
6. Wash with 30 % Ethanol for 5 min 1X.
7. Wash with 1X PBS for 5 min 1X.
8. Put in 0.3 % H₂O₂ for 30 min.
9. Wash with 1X PBS for 5 min 2X.
10. Mark slides using a hydrophobic pen (Darko pen).
D. Incubation with antibodies

1. Place slides in the staining tray (stainless) with moistened kim towel and straw holder.

2. Put within marked area about 1 ml or until full of 5% BSA in PBS.
3. Incubate inside the staining tray for 1 hr. (note: should be covered)
4. Rinse in 1X PBS using a coupling jar.
5. Mark again with hydrophobic pen.
6. Put 1st antibody at 1:2,500 dilution in blocking buffer.
7. Incubate overnight at 4°C.
8. Wash with 1X PBS for 5 min 2X.
9. Add 2nd antibody [Histofine: MAX-PO(MULTI)] and incubate for 1 hr.
10. Wash with 1X PBS for 5 min 2X.
11. Add substrate (Histofine: Simple stain AEC). (note: substrate should be added and viewed in the microscope immediately to monitor development of the reaction).
12. Stop reaction when necessary by rinsing in distilled H$_2$O.
13. Counter stain with hematoxylin for 5 to 15 min.
14. Rinse with tap H$_2$O.
15. Air dry for about 1 hr.
16. Mount slides with cover slip using glycerol and nail polish to seal the sides.

E. Taking pictures

1. Use the microscope with camera attachment.
2. Turn on microscope then the digital camera.
3. Set microscope light in the brightest.
4. Adjust contrast as desired.
5. Use camera LCD to focus pictures.
6. Take pictures at 20X, 40X full, 40X half, 100X full, 100X half or whatever is desired. (note: pictures taken in 100X usually shows the rings formed by high magnification).
7. Transfer pictures to computer using Compact Flash and a PCMCI adapter.
Chapter IV. Bioinformatics

Alignment using ClustalX 1.81

Methods

1. Create input data in Notepad program

1. Create the input data (FASTA format) by using the Notepad program. Follow the pattern below. Each line should be terminated by the “Enter” button.

>gene1 name  (no space and should not be more than 10 characters)
the amino acid sequence of gene 1  (can be both of upper or lower case)

>gene2 name
the amino acid sequence of gene 2

2. Save the input data file.

2. Align with ClustalX program

1. Open the ClustalX program and load the input data file.
2. At the taskbar, click “Alignment” then click at the “Output Format Options”. Mark the clustal and phylip formats.
3. At the taskbar click “Alignment” then click “Do Complete Alignment”. Before processing, the program will show you first the directory where your output files (xxx.aln) will be saved.
4. Close the ClustalX program.

Phylogenetic analysis using MEGA3 and Phylip

Methods

MEGA 3 in combination with ClustalX

Download MEGA 3 package from http://www.megasoftware.net/mega.html
1. Create input data in Notepad program

1. Create the input data (FASTA format) by using the Notepad program. Follow the pattern below. Each line should be terminated by the “Enter” button.

   >gene1 name  (no space and should not be more than 10 characters) the amino acid sequence of gene1 (can be both of upper or lower case)
   >gene2 name
   the amino acid sequence of gene2

2. Save the input data file.

2. Align with ClustalX program

1. Open the ClustalX program and load the input data file.
2. At the taskbar, click “Alignment” then click at the “Output Format Options”. Mark the clustal and phylip formats.
3. At the taskbar click “Alignment” then click “Do Complete Alignment”. Before processing, the program will show you first the directory where your output files (xxx.aln) will be saved.
4. Close the ClustalX program.

3. Convert .aln file to MEGA format

1. At the task bar, click Format button and choose file to convert. Save converted file.

4. Analyze data

1. Close Format window then go to File button and click Open Data. Choose data file to open then click Open button at the window.
2. An Input Data window will pop-out then select data type e.g. Protein Sequence. Click OK button and another window will open to show sequence alignment.
3. Minimize Sequence Alignment window and start analysis using e.g. Phylogenetic analysis routines.

Phylip combination with Clustal X

A. Create input data in notepad program

1. Create the input data (FASTA format) by using the Notepad program. Follow the pattern below. Each line should be terminated by the “Enter” button.

>gene1 name (no space and should not be more than 10 characters)
the amino acid sequence of gene1 (can be both of upper or lower case)
>gene2 name
the amino acid sequence of gene2

2. Save the input data file.

B. Align with ClustalX program

1. Open the ClustalX program and load the input data file.
2. At the taskbar, click “Alignment” then click at the “Output Format Options”. Mark the clustal and phylip formats.
3. At the taskbar click “Alignment” then click “Do Complete Alignment”. Before processing, the program will show you first the directory where your output files (xxx.aln, xxx.dnd and xxx.phy) will be saved. Make sure you save them under the Phylip program folder.
4. Close the ClustalX program.

C. Run the Seqboot program in Phylip

1. Open the window explorer program and explore the Phylip folder.
2. Open or double click the Seqboot icon. The DOS window will be shown. Type the file xxx.phy and press “Enter” to analyze the data. Follow the “answer to questions” below:

“Random number seed (must be odd)?
* (type “1” and enter)

“Are these settings correct? (type Y or the letter for one to change)”
* (type “Y” then the computer will process your data)

3. On window explorer program under the Phylip folder, find and rename the “outfile” to “xxx.boot”.

A. Run the Prodist program in Phylip

1. On window explorer program, double click the Prodist icon. Type the file xxx.boot, and press “Enter” to analyze the data. Follow the “answer to questions” below:

   “Are these settings correct? (type Y or the letter for one to change)”  
   *(type “M” to operate the multiple analysis)

   “How many data sets?”  
   *(type “100”)

   “Are these settings correct? (type Y or the letter for one to change)”  
   *(type “Y” then the computer will process your data)

2. On window explorer program under the Phylip folder, find and rename the “outfile” to “xxx.prot”.

B. Run the Neighbor program in Phylip

1. On window explorer program, double click the Neighbor icon. Type the file xxx.prot, and press “Enter” to analyze the data. Follow the “answer to questions” below:

   “Are these settings correct? (type Y or the letter for one to change)”  
   *(type “M” to operate the multiple analysis)

   “How many data sets?”  
   *(type “100”)

   During this step, you can type “N” to choose the tree methods, either the neighbor-joining or UPGMA method.

   “Are these settings correct? (type Y or the letter for one to change)”  
   *(type “Y” then the computer will process your data)

2. On window explorer program under the Phylip folder, find and rename the “treefile” to “xxx.nei”.
C. Run the Concense program in Phylip

1. On window explorer program, double click the Concense icon. Type the file, xxx.nei, and press “Enter” to analyze the data.

   “Are these settings correct? (type Y or the letter for one to change)” *(type “Y” then the computer will process your data)*

2. On window explorer program under the Phylip folder, find and rename the “treefile” to “xxx.con”.

G. Run the Treeview program

1. Open the Treeview program.
2. Import the xxx.con file from Phylip folder. Use the “all files” option to see and open the xxx.con file. Your tree will be shown by Treeview.

Semi-quantitative PCR using the ImageJ program

Methods

A. Measuring light intensity

1. Download the free ImageJ program from the internet (http://rsb.info.nih.gov/ij/download.html) and install it in the computer.
2. Open the ImageJ program.
3. Select a tool for marking e.g. rectangle or circle.
4. Import the picture you want to analyze thru the Open File menu.
5. Another window will open and show the picture.
6. Mark the area (bands) that you want to measure.
7. Click “measure” at the Analyze menu or press Ctrl M.
8. A 3rd window will appear. The value under the Mean column is the mean light intensity measurement of the selected region.
9. Proceed with other measurements by going to the picture window, selecting another region and pressing Ctrl M. The next measurement will be shown following the first.

B. Computing for relative expression

1. After measuring the bands including the internal control, compute for the relative expression of the gene of interest using the following formula:
gene of interest intensity – background intensity
relative expression =  

2. Get the relative expression values for each of the replicates (at least 3).
3. Compute for significance using statistical tools.

Some useful web resources

2. N-glycosylation site prediction: NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/)
5. Transmembrane regions: Phobius (http://phobius.cgb.ki.se/) and TMpred (http://www.ch.embnet.org/software/TMPREDform.html)
7. Sequence submission: DNA Data Bank of Japan (DDBJ) (http://sakura.ddbj.nig.ac.jp/)
8. Genome Browser: (http://www.ensembl.org/index.html)
Molecular Biology Protocols for Aquatic Organisms

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