Chapter 11

Extraction of DNA and RNA

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Procedure

1. Inoculate the bacterial culture in non-selective enrichment broth viz., TSB or BHI or NB or LB and incubate overnight at optimum temperature (may vary with your bacterial culture).
2. Take 1 mL of overnight grown bacterial culture in 1.5mL centrifuge tube. Label the tubes with the sample id.
3. Centrifuge it at 8000rpm for 5 min.
4. Discard the supernatant.
5. Resuspend the pellet in 0.5mL of 1X Tris-EDTA buffer (pH 8.0) using a vortex or micropipette.
6. Repeat the centrifugation step at 8000rpm for 5 min.
7. Discard the supernatant. To the pellet, add 0.5 ml of Tris-EDTA buffer
8. Resuspend the cells using a vortex or micropipette.
9. Heat the tubes either by placing them in a heating plate or in a water bath at 95°C for 5 minutes.
10. Immediately transfer the tubes to ice or -20^{0}C (if not used immediately).
11. Incubate on ice for 10 min.
12. Centrifuge at 8000 rpm for 5 minutes.
13. The supernatant can be used as template for PCR reactions.

1. Cell lysate preparation from bacterial colonies

Procedure

1. Use micropipette to add 1ml of sterile DNase-free water to the tube.

2. Use a sterile pipette tip to 'touch' a series of bacterial colonies on plate or overnight grown culture on slants and mix it with the DNase free water in the tubes. Avoid picking up a large amount of bacterial cells. Too much cellular material might inhibit the PCR reaction.
3. Centrifuge at 8000 rpm for 5 minutes and discard the supernatant.
4. Add 0.5 ml of Tris-EDTA buffer to the pellet.
5. Resuspend the cells using a vortex or micropipette.
6. Heat the tubes either by placing them in a heating plate or in a water bath at 95°C for 5 minutes.
7. Immediately transfer the tubes to ice or -20^{0}C (if not used immediately).
8. Incubate on ice for 10 min.
9. Centrifuge at 8000rpm for 5 minutes.
10. The supernatant can be used as template for PCR reactions.

2. DNA extraction from fish tissue

CTAB METHOD

1. Collect 100–200 mg tissue in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-lauryl sarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml–1 proteinase K added just before use)
2. Using a disposable stick, homogenize the tissue in the tube thoroughly
3. After homogenization, incubate at 65°C for 1 hour.
4. Add 5 M NaCl to a final concentration of 0.7 M. Next, slowly add 1/10 volume of N-cetyl N,N,N trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly.
5. Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 g for 5 minutes and then transfer the aqueous

solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol.

6. Mix gently and centrifuge at 13,000 g for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice.
7. Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24:1) and centrifuge at 13,000 g for 5 minutes.
8. Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80° C for 15 minutes.
9. Centrifuge at 13,000 g for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilized double-distilled water at 65°C for 15 minutes. Use 1 µl of this DNA solution for one PCR.

3. Dneasy blood & tissue kit (qiagen)

1. Cut tissue into small pieces, and place in a 1.5 ml micro centrifuge tube.
2. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 56°C until completely lysed. Vortex occasionally during incubation.
3. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
4. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing
5. Pipette the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
7. Place the spin column in a new 2 ml collection tube; add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.

8. Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube.
9. Elute the DNA by adding 100 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.

4. RNA extraction from fish tissue

The sample size depends on the type of sample (the following steps shall be performed at a temperature under 4° C):

(1.1) for large (juvenile or adult) shrimp, take 25-75 mg shrimp tissue and add 150 µL trizol. Crush, and then add 850 µL trizol for a final volume of 1,000 µL.

(1.2) For PL shrimp, take 300 shrimp and add 1,000 µL trizol. Crush, then take just 150 µL of the mixture and add 850 µL trizol for a final volume of 1,000 µL.

(1.3) if the sample is haemocytes, add 750 µL trizol and mix for 20 seconds.

(2) Incubate the sample from (1) at 25°C for 5 minutes.

(3) Centrifuge at 12,000g at 4°C for 10 minutes; pipette up the supernatant and transfer it to a new micro centrifuge tube.

(4) Add 200 µL chloroform and mix for 20 seconds.

(5) Incubate at 25° C for 10 minutes.

(6) Centrifuge at 12,000g at 25°C for 10 minutes; pipette up the supernatant and transfer it to a new micro centrifuge tube.

(7) Add 670 µL iso-propanol and mix.

(8) Incubate at 25°C for at least 10 minutes or incubate at -20°C over night or at -70°C for 1 hour.

(9) Centrifuge at 12,000g at 25°C for 10 minutes: pipette off the supernatant and discard.

(10) Rinse the pellet with 0.5 ml of 70% ethanol for atleast 30 minutes at 25°C.

(11) Centrifuge at 12,000g at 25°C for 10 minutes. Pipette off the supernatant and discard.

(12) Leave at room temperature for 20 minutes or until the pellet is dry

(13) Add 150 µL EDTA for every 50 mg of original sample tissue, or if the original sample was 250 µL haemocyte, add 75 µL EDTA. Incubate at 55°C for 15 minutes. Mix gently and use for the next step to create complementary DNA.

5. cDNA synthesis from extracted RNA

This protocol is specifically used for Revert Aid H minus First Strand synthesis kit from Thermo Fisher Scientific

Add total RNA (RNA extracted using the Trizol method) (1 µg to 5 µg- up to 4µL) in a sterile nuclease free, 0.2 mL PCR tube

Add 1 µL of Primer; oligo (dT) 18 primer or random hexamer primer. If gene-specific primer is used add to a final concentration of 15-20 pmol.

Add RNase-free Water to a final volume of 12 µL. Gently flick the tube a few times to mix, and then spin briefly (~5 seconds).

Incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.

Add the following components in the indicated order:

5X Reaction Buffer	4 µL
Ribo Lock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
Revert Aid H Minus M-MuLV Reverse Transcriptase (200 U/µL)	1 µL
Total volume	20 µL

5. For oligo (dT) 18 or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C.

Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C.

6. Terminate the reaction by heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

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