3020BPS Techniques in Molecular Microbiology

Why study microbes?

- Microbes have existed on earth for over 3 billion years
- Microbes genetically are far more diverse than eukaryotes
- Life on this planet as we know it would not have existed without microbes
- Less than 1% of the microbial diversity that exists on earth has been cultured
- Microbes make up one of the single most important source for the biotechnology industry

Given the importance of microbes on our planet (and perhaps on other planets e.g. Mars), the identification and characterisation has become enormously important. Modern, effective and complete identification and characterisation of microbes requires that one is conversant in both culturing and molecular techniques. This course enables students to identify and differentiate anaerobic microbes using conventional culturing techniques, microscopy and modern molecular / phylogenetic approaches.

Though 3020BPS Techniques in Molecular Microbiology is essentially a laboratory-based project important theory behind some the laboratories are discussed. The course is designed for students in the Bachelor of Science with a major in Microbiology, Bachelor of Biotechnology or a Science degree with some background in microbiology. The laboratory is designed to increase competence and confidence of students in the laboratory and thereby enable students to use such skills to complete more complex laboratory tasks either in research or in routine situations. The laboratory runs as a project in which a taxonomically uncharacterised bacterial isolate is studied, using a range of techniques widely used in diagnostic and research microbiology laboratories. Students are required to set up and run experiments and submit a written report. The findings are also presented as an oral scientific paper in the presence of their fellow students. The fellow student audience is encouraged to participate by asking questions and critically evaluating the presentation.

The laboratory provides skills for developing careers in microbiology research or in allied industries such as biotechnology, waste water, food and water, public health and hospitals. It uses a number of techniques used for the identification and taxonomic characterisation of unknown microorganisms. These techniques can be applied in many microbiological laboratories across a broad range of areas.

Aim:

To characterise an unknown and unidentified thermophile anaerobe from a deep subsurface high temperature environment or a halophilic anaerobe from salt lake sediments.

Background:

You will be provided with the following information during the lectures and labs:

Theory behind experimental methods:

1. Environments, culture-dependent & culture-independent methods
2. Anaerobes and culturing anaerobes
3. DNA extraction
4. 16S rRNA genes.
5. PCR and Sequencing
6. Sequence analysis and software
    - Bioedit
    - TreeCon
    - Cutoff
    - Ribosomal Database Project (RDP)
    - Blast

Writing your Report:

1. Appropriate referencing
2. Organisation of data and report sections
3. The role of Bergeys Manual Trust
4. International Journal of Systematic and Evolutionary Microbiology as a clearing house for new taxa

Overview of the methods:

A pure isolate will be characterised including morphology studies (gram-staining and electron microscopy), growth studies (growth effect of temperature, pH, NaCl and antibiotics), nutritional studies (electron acceptors and electron donors) and 16S rRNA gene sequencing and analyses.

Composition of Media

Medium: Tryptone – Yeast Extract – Glucose (TYEG) Media

TYEG media was previously formulated for the isolation and growth of chemoheterotrophic anaerobes and consists of a basal salts medium (low phosphate buffered salts) amended with tryptone, yeast extract and glucose. The medium is prepared anaerobically and contained (per litre of distilled water):

Basal salts - Low Phosphate Buffered Salts (LBPS)

Component	*Concentration (g/L)*
$MgCl_2.6H_2O$	0.2
NH_4Cl	0.9
KH_2PO_4	0.75
K_2HPO_4	1.5
NaCl	0.9
10% $FeSO_4$	5 µl
Zeikus' Trace Element Solution	9 ml
Wolin's Vitamin Solution	5 ml
0.2% Resazurin	1 ml
10% $Na_2S.9H_2O$[#]	2 ml
dH_2O	To 1 l

[#] $Na_2S.9H_2O$ is a reducing agent and is added after the medium is boiled. $Na_2S.9H_2O$ is not included in media used to test electron acceptor utilisations.

The addition of 2 g/l yeast extract, tryptone and glucose will be made to the appropriate tubes in the preparation of media for individual tests.

Laboratory 1 - Making and Dispensing Media (week 2)

A. Preparing stock solutions.

Prepare 10 % (w/v) solutions of glucose, tryptone, yeast extract, sucrose, xylose, Na acetate, Na propionate, Ma malate, glycerol, casamino acids and peptone. Prepare 1 M solutions of Na sulphate, Na thiosulfate and Na nitrate. Prepare 0.5 M solution of sodium sulphite.

B. Preparing Hungate tubes prior to dispensing media.

a. *Media for testing **electron acceptor** utilisation tests* (7 tubes)

i. Add 0.02 g of Ammonium Ferric Citrate to an empty hungate tube and label Fe(III)
ii. Add 0.015 g of MnO_2 to an empty hungate tube and label Mn(IV).
iii. Add 200 µl of 1 M Na sulfate and label sulfate.
iv. Add 200 µl of 0.5 M Na sulfite and label sulphite.
v. Add 200 µl of 1 M Na thiosulfate and label thiosulfate.
vi. Add 200 µl of 1 M Na nitrate and label nitrate.
vii. Add 0.1 g of sulfur and label sulfur.

Once the above tubes have been prepared, add 200 µl of 10 % yeast extract to each of the tubes.

b. *Media for testing **electron donors*** (8 tubes)

i. Add 200 µl of 10 % sucrose and label sucrose.
ii. Add 200 µl of 10 % xylose and label xylose.
iii. Add 200 µl of 10 % acetate and label acetate.
iv. Add 200 µl of 10 % propionate and label propionate.
v. Add 200 µl of 10 % malate and label malate
vi. Add 200 µl of 10 % glycerol and label glycerol.
vii. Add 200 µl of 10 % casamino acids and label casamino acids.
viii. Add 200 µl of 10 % peptone and label peptone.

c. *Media for testing **salinity*** (4 tubes)

i. Add 0.1 g NaCl to an empty hungate tube and label 1 % NaCl.
ii. Add 0.2 g NaCl to an empty hungate tube and label 2 % NaCl.
iii. Add 0.3 g NaCl to an empty hungate tube and label 3 % NaCl.
iv. Add 0.4 g NaCl to an empty hungate tube and label 4 % NaCl.

d. *Media to test different **pH*** (6 tubes)

i. Add 333 µl of 1 M HCl to an empty hungate and label pH 4.
ii. Add 225 µl of 1 M HCl to an empty hungate and label pH 5.
iii. Add 145 µl of 1 M HCl to an empty hungate and label pH 6.
iv. Add 75 µl of 1 M NaOH to an empty hungate and label pH 8.
v. Add 155 µl of 1 M NaOH to an empty hungate and label pH 9.
vi. Add 300 µl of 1 M NaOH to an empty hungate and label pH 10.

e. *Label tubes for **temperature** studies* (6 tubes)

i. Label the tube as temp1
ii. Label the tube as temp2
iii. Label the tube as temp3
iv. Label the tube as temp4
v. Label the tube as temp5
vi. Label the tube as temp6

f. *Label tubes for* ***antibiotic*** *studies* (5 tubes)

i. Label the tube as ant1
ii. Label the tube as ant2
iii. Label the tube as ant3
iv. Label the tube as ant4
v. Label the tube as ant5

g. *Label tubes as* ***spare*** *tubes* (6 tubes)

i. Label the tube as spa1
ii. Label the tube as spa2
iii. Label the tube as spa3
iv. Label the tube as spa4
v. Label the tube as spa5

C. Preparing Media

i. In a large beaker prepare 500 ml of LPBS amended with 0.2 g / l yeast extract. Transfer to a 1 l Schott bottle and set the pH to 7.2 – 7.4. (NOTE - at this stage do not include the tryptone, glucose, resazurin or the $Na_2S.9H_2O$ in the media).

ii. Thoroughly, boil the medium to exclude oxygen, gas the head with oxygen-free nitrogen and attach the dispenser. NOTE – this will be demonstrated to you.

D. Dispensing Media

All media is dispensed in 9 ml aliquots under oxygen-free nitrogen gas. (This will be demonstrated to you).

Part 1. Dispense media into tubes containing the electron acceptors.
Dispense 9 ml aliquots into the Hungate tubes prepared for electron acceptor tests.

Part 2. Dispense media into tubes containing the electron donors.
Add 0.2 ml of 10 % $Na_2S.9H_2O$ to the remaining media and dispense into the Hungate tubes containing electron donors.

Part 3. Dispense media for growth studies (salinity, pH, temperature, antibiotics and spare).
To the remaining medium, add 7.5 ml each of 10% yeast extract, 10% tryptone and 10% glucose to give a final concentration of 0.2% of each carbon source per tube.

Dispense 9 ml of the medium into Hungate tubes labelled for salinity, pH, temperature and antibiotics

Laboratory 2 - Inoculations and incubations (week 3)

INOCULATIONS

A fresh culture will be provided to you. 0.4 ml of inoculum will be used for all media transfers. As all of the isolates are anaerobes, all transfers and inoculations are to be performed using needle and syringe. This will be demonstrated to you.

INCUBATIONS

pH, NaCl antibiotic susceptibility, electron donor and acceptor tests will be incubated at the temperature in which the strains were isolated (will be advised).

1. Temperature studies

The isolate will be inoculated into tubes of TYEG media and incubated at different temperatures for the determination of the isolates temperature optima and range. Unless advised differently the temperatures tested will include: 30, 40, 50, 55, 60, 65, 70.

2. pH and Salinity studies

This media is already prepared and labelled, pH (4 – 10), NaCl (0.1 %, 1 – 5%). Just inoculate and incubate.

Note: if a strain's incubation temperature is 50 °C, the tube at 50 °C used for the temperature study can be used as a pH 7.0 and NaCl 0.1%.

3. Antibiotic susceptibility

Using a needle and syringe, add 0.1 ml of 10 μg / ml stocks of ampicillin, tetracycline, chloroamphenicol, streptomycin and penicillin to tubes of TYEG to give a final antibiotic concentration of 10 μg ml^{-1}.

4. Electron donor and acceptor tests

Inoculate the electron acceptors, electron donors and electron donor control. Incubate at specific temperature.

5. OD readings

Take time 0 readings [OD (580nm)] for all cultures with the exception of electron acceptors. This will be demonstrated.

6. Gram staining

Gram staining will be used to determine both the Gram reaction and the cellular morphology of your isolate. Further morphological studies will be done with the electron microscopy in week .

- Prepare a smear of the bacterial culture, air dry and heat fix.
- Apply crystal violet to just cover the smear and leave for 1 minute.
- Wash gently with water and shake to remove excess water.
- Flood the smear with iodine solution and leave for 1 minute.
- Wash gently with water and shake to remove excess water.
- Add alcohol and gently rock the slide, tip off alcohol and repeat.
- Again wash with water and shake to remove excess water.
- Stain with safranine for 10 – 20 seconds.
- Wash with water and **gently** blot dry taking care not to rub the smear off the slide.
- Examine microscopically under oil immersion.
- Gram positive bacteria stain violet-purple and Gram negative bacteria pink.

Laboratory 3 – Measurements (week 4)

Measuring growth
The OD (580nm) of all cultures will be read after the relevant incubation period, as demonstrated. Do any experiments need to be repeated?

Measuring reduction of a sulfate, sulfite, elemental sulfur and thiosulfate
Make a 10 % stock solution of $Fe(NH_4)_2(SO_4)_2$. Add 0.5 ml into the sulfate, sulfite, elemental sulfur and thiosulfate tubes. Tubes are scored as positive for the reduction of oxidized sulfur compounds if black FeS is formed.

Why do we need an assay to measure reduction of the oxidized sulfur compounds? Why can we not just measure growth?

Fe(II) and Mn(IV) reduction
Fe(III)-reduction will be inferred when a transformation of the reddish-brown colour of the Fe(III) oxide to a dark precipitate [Fe(II)] and a clearing of the media is observed. Mn(IV) reduction is inferred by a clearing of the media.

Laboratory 4 –Electron Microscopy (weeks 7 & 8)

In the laboratory session before the scheduled electron microscopy, inoculate a spare tube of medium with your unknown bacterium and incubate.

Preparation and viewing of the cultures will be at the EM unit at QUT. Dr Deb Stenzel will take you for this session. You will to go directly to QUT Gardens Point campus, Block R, level 1.

EM sessions will take place in either week 7 or 8 Time and further details will be given closer to the time.

Laboratories – Molecular Microbiology (weeks 6-9)

Overall plan of 16S rRNA gene sequencing and sequence Analysis:

1. Prepare chromosomal DNA from bacterial cultures.
2. Check PCR products on 1% agarose gel.
3. PCR amplify 16S rRNA gene using universal amplification primers (Fd1 and Rd1).
4. Clean up PCR products using QIAGEN PCR clean-up spin columns.
5. Sequence purified PCR products using universal 16S sequencing primers.
6. Clean up sequencing reactions and submit to Molecular Biology Facility.
7. Analyse sequences using RDP and Bioedit.

1.Preparation of Genomic DNA from Bacteria

Stock Solutions and Materials:

1. P1 Buffer from QIAGEN kits. It contains RNAse
2. 10 mg/ml achropeptidase *[Sigma]*
3. 50 mg/ml lysozyme *[Sigma]*
4. 10% (w/v) sodium dodecyl sulfate (SDS)
5. 20 mg/ml proteinase K *[Fermentas]*
6. Chloroform:isoamylalcohol + phenol (1+ 1 to give a final ratio of 24:1:25)
7. 100 % ethanol, ice cold

Method:

1. Transfer a log phase growing culture to a red-capped 15 ml centrifuge tube and concentrate by centrifuging at 7 500 rpm for 10 minutes.

2. Decant the supernatant and drain well onto tissue.

3. Resuspend by repeated pipetting the pellet in 467 µl P1 buffer.

4. Add 8 µl of lysozyme (50 mg/ml), 15 µl of achromopeptidase (6 mg/ml) to give final concentrations of 0.8 mg/ml and 0.3 mg/ml respectively.

5. Gently mix by taping and incubate at 37 ^{o}C for 1h.

6. Add 30 µl of 10% SDS, 3 µl of 20 mg/ml proteinase K, mix, & incubate 1 hr at 50 °C.

7. Add an equal volume of chloroform:isoamylalcohol:phenol (24:1:25) and mix by GENTLE INVERSION for at least 10 mins so as to mix the phases. **CAUTION: PHENOL CAUSES SEVERE BURNS, WEAR GLOVES GOGGLES, AND LAB COAT AND KEEP TUBES CAPPED TIGHTLY.**

8. Spin in a micro centrifuge at 12,000 RPM for 10 min.

9. Transfer the upper aqueous phase to a new microfuge tube.

NOTE: Repeat steps 8, 9 and 10 ONLY if necessary.

10. Add an equal volume of ice cold 100% ethanol
11. Spool DNA onto a glass rod (or Pasteur pipet with a heat-sealed end) and resuspend in a small amount (100 ul) of ethanol.

OR

12. If DNA does not spool, centrifuge for 15 mins, and decant the ethanol.
13. Evaporate the remaining ethanol at room temperature or at 50 ^{o}C.
14. Dissolve the DNA pellet in 40 µl TE buffer. If difficult to resuspend, incubate at 50 ^{o}C for 15 to 30 mins.
15. After DNA has dissolved, determine the concentration by agarose gel electrophoresis (Refer to section 2).

Reagents required:

TE buffer:

10 mM Tris-HCl (pH, usually 7.4 or 8.0)
1 mM EDTA (pH 8.0)
Use concentrated stock solutions to prepare. If sterile water and sterile stocks are used, there is no need to autoclave. Otherwise, sterilize solutions by autoclaving for 20 minutes. Store the buffer at room temperature.

1 M Tris-Cl – used at various pHs

Using Tris base: To make 1 liter, dissolve 121 g Tris Base in 800 ml of water. Adjust pH to the desired value by adding approximately the following:
pH = 7.4 about 70 ml of concentrated HCl
pH = 7.6 about 60 ml of concentrated HCl
pH = 8.0 about 42 ml of concentrated HCl
Make sure solution is at room temperature before making final pH adjustments. Bring final volume to 1 liter. Sterilize by autoclaving.

SDS stock- Use a mask when weighing this out.

10% or 20% (w/v) SDS. Also called sodium lauryl (or dodecyl) sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68 ° C and stir with a magnetic stirrer to assist dissolution. If necessary, adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H 2O. Store at room temperature. Sterilization is not necessary. Do not autoclave.

3M Sodium Acetate - pH 5.2

To prepare a 3 M solution: Dissolve 408.3 g of sodium acetate-$3H_2O$ in 800 ml of H 2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

Isoamyl alcohol Chloroform Mixture – (DONOT STERILISE)

Lysozyme – (DONOT STERILISE)

Achromopeptidase - (DONOT STERILISE)

Phenol – (DONOT STERILSE)

Extract to the Max: Phase Lock Gel

Phase Lock Gel (PLG) is a unique product that eliminates interface-protein contamination during phenol extraction. It guarantees faster results with improved recoveries. PLG is a proprietary compound that, upon centrifugation, migrates to form a tight seal between the phases of an aqueous/organic extraction. The organic phase and the interface material are effectively trapped in or below the barrier.

This allows the complete, easy transfer of the entire aqueous phase by simply decanting or pipetting, resulting in increased yields of up to 30%. In addition, the risk of contaminating the sample with interface material is eliminated. PLG also offers increased protection from exposure to hazardous compounds. Phase Lock Gel can be adapted to virtually any protocol requiring extraction of an aqueous sample with phenol and/or chloroform[1]. PLG is provided predispensed into standard centrifuge tubes of various sizes for your convenience.

[1]Murphy, N.R. and Hellwig, R.J. 1996 BioTechniques 21(5): 934-939.

2. Agarose Gel Electrophoresis.

Materials:
Electrophoresis grade agarose (low EEO) [DNA grade agarose]
1×TAE running buffer (or 1×TBE)
Ethidium bromide (5 mg/mL**)** **{Extreme (poison), May cause heritable genetic damage}**
Agarose sample dye

Method:

1. For 0.7% agarose gel (per 50mL), 0.35g agarose, 50 mL 1× TAE buffer.
2. Boil for 1 minute and allow to cool to ~50°C.
3. Add 5 µL of 5 mg/mL Ethidium bromide.
4. Mix by swirling and pour into prepared casting rig.
5. Allow gel to set (until gel turns opaque, approximately 15 minutes).
6. Place gel in electrophoresis apparatus and submerge with 1×TAE running buffer.
7. Prepare samples by mixing loading dye with DNA sample on the opaque/translucent side of a strip of paraffin, usually 2 µL loading dye: 5-10 µL DNA sample.
8. Load samples and appropriate DNA standards (typically λ*Hin*d III linear standards) into gel wells. λ*Hin*d III is stored in the fridge.
9. Electrophoresis at 120V, 80 mA, for 40 minutes.
10. Visualise bands under U.V. light.

Notes:

Disposable gloves must be worn at all time when handling casting rig, gel and anything that comes into contact with Ethidium bromide. Separate conical flasks are used for gel preparation and separate pipettes are used for dispensing Ethidium bromide and loading samples.

3. **PCR Amplification of Bacterial 16S rRNA Genes.**

Materials:

Sterile dH_2O (dH_2O)
dNTP mix (25 mM)
Forward primer (Fd1; 50 µM)
Reverse primer (Rd1; 50 µM)
Taq reaction buffer ($MgCl_2$ free, ×10)
$MgCl_2$ (25 mM stock)
Taq polymerase (5U/µL)
Sterile mineral oil

Method:

1. To a **0.6 mL** thin-walled microcentrifuge tube, prepare 2 µL aliquots of template DNA as follows:
   - No DNA control (i.e. 2 µL sdH_2O)
   - Neat (2 µL of chromosomal DNA)
   - 1:50 (2 µL of chromosomal DNA diluted 1 in 10 with sdH_2O)
   - 1:100 (2 µL of chromosomal DNA diluted 1 in 50 with sdH_2O)

   (Prepare dilutions in sdH_2O)

2. Prepare a master mix of the remaining PCR reagents in a **1.5** mL microcentrifuge:
   - The components of the master mix should be calculated for a number of reactions one larger than the number being set up (i.e. in this case, a total of five).
   - Single reaction and master mix (example):

1 reaction	Master Mix	Reaction component
1.0 µL	5.0 µL	50 µM Fd1
1.0 µL	5.0 µL	50 µM Rd1
0.5 µL	2.5 µL	25 mM dNTPs
5.0 µL	25.0 µL	10× *Taq* buffer
4.0 µL	20.0 µL	$MgCl_2$
36.3 µL	181.5 µL	sdH_2O
0.2 µL	1.0 µL	*Taq* polymerase (5 U/µL; 1 U per reaction)

3. Place 48 µL of the master mix into each 0.6 mL tube.

4. Overlay with 50µL of sterile mineral oil.

5. Place tubes in the Corbet Thermal Cycler and run on program 5.

6. At the completion of the PCR program, check a 5µL aliquot (with 2 µL of dye) of each reaction on a 0.7% agarose gel.

7. Store PCR products at -20°C.

The program given below is for the Corbet Thermal Cycler. Link Program 5:

Cycle	Step	Temperature	Time	TTC
1	1	95	2:00	
	2	50	1:00	
	3	70	2:00	1
2	1	94	0:55	
	2	50	1:00	
	3	72	2:00	30
32	1	23		1

4. Clean up PCR products using QIAGEN PCR clean-up spin columns.

- Run all of PCR product (minus the mineral oil) on a 0.7% agarose gel.
- Excise the DNA fragment from the gel using a sterile glass slide cover (or scalpel) over the transilluminator. (UV glasses and the protective UV cover must be used when using this transilluminator. Be careful of the UV rays.)
- See methods in the QIAGEN procedure manual – QIAquick Gel Extraction Kit.

5. ABI Big-Dye Sequencing of 16S rDNA PCR Products.

Materials:

Sterile dH_2O

Sequencing primers (3.2 µM)

10× Big-Dye buffer

Big-Dye Sequencing Reaction Mix (400 µl costs $1200+)

Sterile mineral oil

Method:

1. Prepare a master mix of Sequencing Reagents in a **0.6 mL** microcentrifuge tube containing per single reaction *(Dispense on ice)*:

Sequencing primer*	(3.2 µM) 1µL	*(Make fresh dilute from stock 1:15)*
Big-Dye	4µL	*(Kept in the fridge)*
5× Big-Dye buffer	2µL	*(Kept in the freezer)*
sdH_2O	To 20µL when combined with Template DNA.	

   *For first sequence use only F1 and R5.

2. Combine appropriate of Sequencing Master Mix with appropriate amount (1-4µL) of PCR product to give a final volume of 20µL in a 0.2 ml microcentrifuge tube.

3. Place the sequencing reactions in the Idaho thermal cycler and run the Big-Dye cycler sequencing program (Program #25).

Cycling Conditions

1. 96°C for 1 min
2. 96°C for 10 sec
3. 50°C for 5 sec
4. 60°C for 4 min

Repeat steps 2-4 for 25-30 times and then hold at 4°C until ready to purify.

Template Quality and Quantity

The success of your sequencing reaction depends upon the quality and quantity of DNA template. The capillary system is very sensitive to contaminants such as proteins, RNA and residual salts. Not only is the quality of result affected by such contaminants but the presence of proteins and high concentrations of DNA can reduce the life of the capillary array. For detailed information on DNA template preparation, DNA template quality and quantity, primer design and quantitation, refer to *Performing DNA Sequencing Reactions*, Section 3 in the Applied Biosystem's Automated DNA Sequencing Chemistry Guide (Part# 4305080).

Template Quality: Potential contaminants include proteins, RNA, residual salts, excess PCR primers, dNTPs, enzyme and buffer components (from PCR amplification). The DNA should be examined by agarose gel electrophoresis and spectrophotometry in order to assess the quality of the template.

Template Quantity: Template quantitation is critical for successful sequencing reactions. The preferred method for quantitation is by gel electrophoresis with a DNA mass ladder standard.

Reference: BigDye Terminator v1.1 Cycle Sequencing Kit Protocol, Applied Biosystems 2002 (Part# 4337036A), BigDye Terminator v3.1 Cycle Sequencing Kit Protocol, Applied Biosystems 2002 (Part# 4337035A)

Recommended amount of template and primer to use in a cycle sequencing reaction:

PRIMER	**3.2 pmol**
TEMPLATE:	
PCR product:	
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
1000-2000 bp	10-40 ng
>>2000 bp	20-50 ng
Plasmid, single-stranded	25-50 ng
Plasmid, double-stranded	150-300 ng
Cosmid, BAC	0.5-1.0 µg
Bacterial Genomic DNA	2-3 µg

Reference: Page 2-6 of the Applied Biosytems Sequencing Protocol

Reaction Set-Up

2. Recommended Reaction Set-ups using BDTv1.1/BDTv3.1:

Reaction	**1X**	**1/2X**	**1/4X**	**1/8**
BDT (µL)	8	4	2	1
5X SEQ Buffer (µL)	0	2	3	3.5
Primer (µL)	3.2 pmol	3.2 pmol	3.2 pmol	3.2 pmol
Template	As required	As required	As required	As required
MilliQ Water	**To 20 µL**	**To 20 µL**	**To 20 µL**	**To 20 µL**

Mix well and spin briefly.

6. Precipitation of Sequencing Reaction Products.

Materials:

Sterile EDTA (125mM)

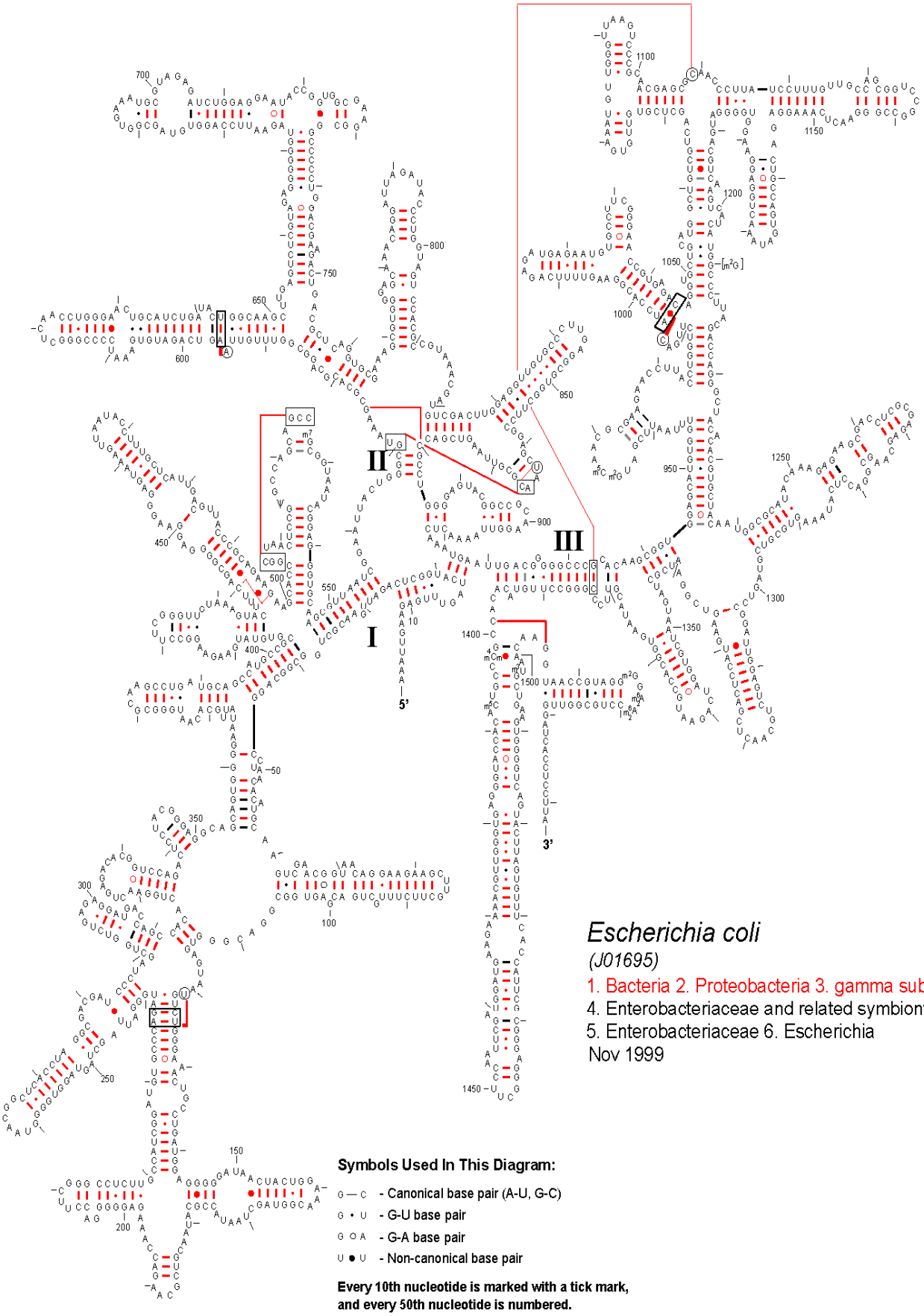
100% Ethanol

70% Ethanol

Method:

1. Add 5 µL 125mM EDTA to each tube.
2. Transfer to 1.5 mL microcentrifuge tube and add 60 µL 100% ethanol.
3. Incubate at room temperature for 15 minutes.
4. Spin at top speed in a microcentrifuge tube for 20 minutes to precipitate the extension products.
5. Carefully discard the supernatant.
6. Wash pellet with 190 µL 70% ethanol.
7. Spin at top speed in a microcentrifuge tube for 10 minutes.
8. Discard supernatant and allow the pellet to dry thoroughly.

Secondary Structure: small subunit ribosomal RNA



Escherichia coli

(J01695)

1. Bacteria 2. Proteobacteria 3. gamma subdivision
4. Enterobacteriaceae and related symbionts
5. Enterobacteriaceae 6. Escherichia

Nov 1999

Symbols Used In This Diagram:

G — C - Canonical base pair (A-U, G-C)

G • U - G-U base pair

G ○ A - G-A base pair

U ● U - Non-canonical base pair

Every 10th nucleotide is marked with a tick mark, and every 50th nucleotide is numbered.

Tertiary interactions with strong comparative data are connected by solid lines.

List of amplification & sequencing primers:

Primer	*E. coli* Position	Sequence 5'→ 3'
Amplification & Sequencing		
Fd1	8 – 27	AGA GTT TGA TCC TGG CTC AG
Rd1	1542 – 1526	AAG GAG GTG ATC CAG CC
Sequencing		
F1	339 – 357	CTC CTA CGG GAG GCA GCA G
F2	785 – 805	CAG GAT TAG ATA CCC TGG TAG
F3	907 – 926	AAA CTC AAA GGA ATT GAC GG
F4	1391 – 1406	TGT ACA CAC CGC CCG T
R1	357 – 342	CTG CTG CCT CCC GTA G
R2	536 – 519	GTA TTA CCG CGG CTG CTG
R3	802 – 785	CCA GGG TAT CTA ATC CTG
R4	926 – 907	CCG TCA ATT CTT TGA GTT TT
R5	1115 – 1100	GGG GTT GCG CTC GTT G
R6	1513 – 1494	TAC GGT TAC CTT GTT ACG AC

Sequencing strategy for 16s rRNA genes

- Primer names and annealing positions are marked
- Arrows indicate direction of sequence read and line lengths indicate amount of sequence generated by a 450 base read
- Sizes are given in base pairs (bp)