2013 BioNanotechnology Summer Institute

Cancer Nanotechnology and Cellular Mechanics

an NSF-IGERT/NIH-CNTC GEM4 collaboration

July 29 - August 9, 2013

University of Illinois at Urbana-Champaign

MOLECULAR BIOLOGY LAB MODULE
Location: 1265 Digital Computing Lab (DCL)
Lead Instructor: Susan Steenbergen, Pathobiology
Lab Assistant: Emily Tillmaand, Neuroscience

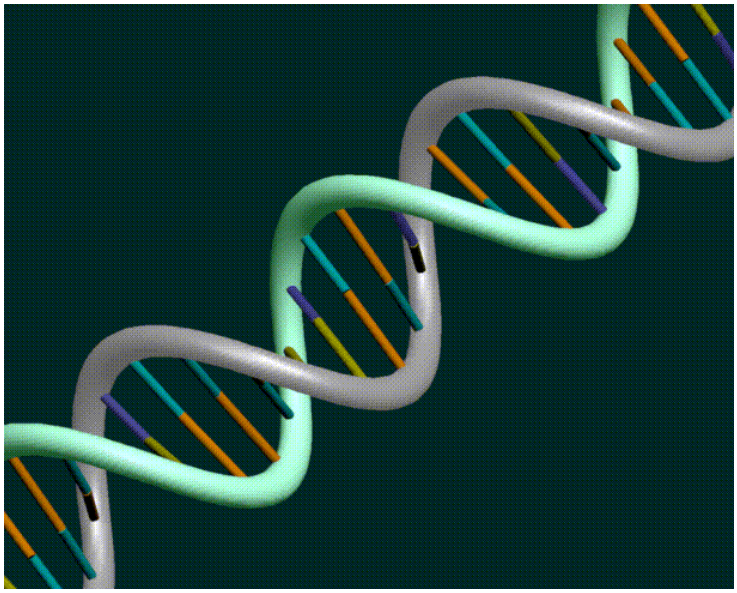
Purpose and Expected Outcome:

In this lab module you will:

- Learn how to isolate genomic DNA from gram negative bacteria
- Perform site-directed mutagenesis on the GFP gene by PCR
- Purify total RNA

Please read the three selected articles taken from Current Protocols in Molecular Biology before attending the lab module sessions:

1. CHAPTER 2 - Preparation and Analysis of DNA
2. CHAPTER 15 - The Polymerase Chain Reaction
3. CHAPTER 4 - Preparation and Analysis of RNA



ISOLATION OF GENOMIC DNA FROM GRAM NEGATIVE BACTERIA

You will be given a 1.5 ml microcentrifuge tube containing a pellet of *Escherichia coli*, strain BW30270. This strain is a K-12 or laboratory strain of *E.coli*. The pellets are made up of cells from 3 mls of overnight growth in a rich medium.

1. Add 600 µl of Nuclei Lysis Solution. Gently pipet up and down until the cells are resuspended.
2. Incubate at 80°C for 5 minutes to lyse the cells; then cool to room temperature.
3. Add 3 µl of RNase Solution to the cell lysate. Invert the tube 2–5 times to mix.
4. Incubate at 37°C for 30 minutes. Cool the sample to room temperature.
5. Add 200 µl of Protein Precipitation Solution to the RNase-treated cell lysate.
6. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate, be sure that the two solutions have completely mixed. Do not over-vortex or you risk shearing the chromosome.
7. Incubate the sample on ice for 5 minutes. Centrifuge at 13,000 rpm for 10 minutes.
8. Transfer the supernatant containing the DNA to a clean 1.5ml micro-centrifuge tube containing 600µl of room temperature isopropanol. Do not carry over any flecks of precipitate.
9. Gently mix by inversion until the thread-like strands of DNA form a visible mass. If you do not see threads but a general whitish appearish mix well and continue.
10. Centrifuge at 13,000 rpm for 5 minutes.
11. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600 µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet. **YOU CAN STOP HERE**

12. Centrifuge at 13,000 rpm for 2 minutes. Carefully aspirate the ethanol from the tube with a pipet. Be careful not to suck up your pellet!
13. Drain the tube on kimwipes and allow the pellet to air-dry for 10–15 minutes.
14. Add 100 µl of sterile water to the tube and rehydrate the DNA. Alternatively you can use 100 µl of a Tris-EDTA buffer.

SITE-DIRECTED MUTAGENESIS BY PCR AMPLIFICATION

The first step in the mutagenesis of the *gfp* gene is to methylate the plasmid which carries the gene. This plasmid, pGLO, has been propagated in *E. coli* and then purified. We have done the methylation for you due to time constraints but the procedure is given below. The methylase used transfers methyl groups from S-adenosylmethionine to cytosine residues occurring next to guanine. When DNA is methylated in this way, and then transformed into a wild-type strain of *E. coli* the DNA will be degraded. Thus after amplification of the plasmid with mutagenic primers, resulting in mutagenized plasmid that is not methylated, only this new plasmid DNA will be replicated in the cell.

Methylation reaction

Mix together:

- 100 ng of the pGLO plasmid
- 1.6 μl of 10X methylation buffer
- 1.6 μl of 10X S-adenosylmethionine (SAM)
- 1 μl DNA methylase
- water to 16 μl total volume

Incubate at 37°C for 1 hour.

<u>YOU WILL BEGIN AT THIS STEP</u>

Mutagenesis reaction

Mix together:

- 4 μl methylated plasmid
- 1 μl of pGLO primer mix
- 45 μl PCR Supermix High Fidelity

Cycling parameters:

- 94° C for 2 min.
- 94° C for 30 sec.
- 55° C for 30 sec.
- 68° C for 6 min.
- back to step 2 for 20 more cycles
- 68° C for 10 min.

After amplification is complete remove 17 μl of the PCR reaction to a clean microfuge tube, add 3 μl of tracking dye and run on an agarose gel to verify amplification. Next, 4 μl of the reaction is transformed into *E. coli* DH5α and plated on a rich media containing ampicillin and arabinose. The ampicillin selects for the plasmid and the arabinose induces the transcription of the GFP gene. It is important to keep the cells on ice and chilled until the heat shock in step 4. Never vortex competent cells.

1. Put 4 μl of each ligation reaction in a sterile 1.5 ml tube and place on ice. Thaw competent cells on ice.
2. Transfer 50 μl of competent DH5α into the 1.5 ml tube on ice and gently mix by tapping the tube.
3. Incubate tubes on ice for 15 min.
4. Heat shock the cells for 3 min. at 42°C.
5. Return tubes to ice for 2 min.
6. Add 900 ml LB broth and incubate at 37°C for 1 hour.
7. Spin culture, remove most of the media, resuspend pellet in remaining media and plate on LB + 100μg/ml Ampicillin + 0.6% Arabinose.
8. The plates will be incubated overnight at 37°C.
9. Using a hand held UV light look at the colonies to see if the *gfp* gene has been inactivated.

PURIFICATION OF TOTAL RNA

We will be preparing RNA from *E. coli* cells, both cells that have been grown in the presence of sialic acid and cells grown in the presence of glycerol.

1. Resuspend the cell pellet in 175 μl of RNA lysis buffer.
2. Add 350 μl SV RNA Dilution buffer. Mix by inverting 3-4 times. Incubate at 70°C for **3** minutes (no longer).
3. Centrifuge for 10 minutes at 13,000 rpm, transfer cleared lysate to a fresh tube.
4. Add 200 μl 95% ethanol and mix well.
5. Assemble a spin basket assembly. Transfer mix from step 4 to the assembly and centrifuge for 1 min. Discard fluid.
6. Add 600 μl of SV RNA wash solution (ethanol added). Centrifuge for 1 min, discard fluid.
7. Apply 50 μl DNase mix to the membrane. Incubate at RT for 15 min.
8. Add 200 μl SV DNase Stop solution, centrifuge 1 min.
9. Add 600 μl SV RNA Wash solution, centrifuge 1 min., discard fluid.
10. Add 250 μl SV RNA wash solution and centrifuge for 5 min. Transfer spin basket to elution tube.
11. Add 100 μl Nuclease-Free Water to membrane. Centrifuge for 1 min. to elute RNA. Place on ice immediately.

REAL TIME PCR REACTIONS

We will not be setting up the qRT-PCR reactions due to time but we will be look at results that demonstrate how this method is used to quantify the amount of message present in a sample. The results show amplification of a transcript of the Aujeszky's Disease virus from RNA purified from the cells you used above. In order to verify the quantification amplification is also done of a "house-keeping" gene, ubiquitin. This gene is expressed at a constant rate in cells and so the amount of amplification products that are made should not change from the infected or uninfected cells. Thus, even though one adds equivalent amounts of RNA from infected and uninfected cells to each tube, if the ubiquitin reactions do not give the same result we have a way to normalize the two different RNA samples. It is important to add 2 controls, a control with no reverse transcriptase added, to insure our amplification products are not coming from DNA, and a no template control, to insure none of the reagents are contaminated. The results we will discuss are from amplification reactions done with the Promega GoTaq 1-step RT-qPCR system. The table below shows the set up used to prepare the reactions. While we will not set up these reactions due to time take note of the controls set up for each set of primers as well as the test samples.

In order to get in all the controls necessary 6 tubes are set up in the manner below.

	tube 1	tube 2	tube 3	tube 4	tube 5	tube 6
2 X GoTaq qPCR mix (contains reference dye)	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl
Primer mix	2.5 μl of PRV primers	2.5 μl of PRV primers	2.5 μl of PRV primers	2.5 μl of PRV primers	2.5 μl of ubiquitin primers	2.5 μl of ubiquitin primers
RNA template	5 μl of glycerol grown cells	5 μl of sialic acid grown cells	5 μl of glycerol grown cells (no RT)	5 μl of sialic acid grown cells (no RT)	5 μl of glycerol grown cells	5 μl of sialic acid grown cells
GoScript RT Mix	0.4 μl	0.4 μl	-	-	0.4 μl	0.4 μl
Water	2.1μl (add to a final total volume of 20 μl)	2.1μl	2.5 μl	2.5 μl	2.1μl	2.1μl

Samples were run in an ABI7000 with the following cycles.

1 cycle of 95° C for 2 min.

40 cycles of 95° C for 15 sec. (denaturation) followed by 60° C for 1 min. (annealing and extension).

After the amplication cycles are complete the machine does a melting curve to insure that the dsDNA being measured is a real amplification product and not due to primer interaction, so called “primer-dimers”.

Good reference sources for Molecular Biology techniques

Molecular Cloning: A Laboratory Manual, Sambrook and Russell, Cold Spring Harbor Press.

Current Protocols in Molecular Biology, Ausubel et al editors, Wiley Interscience

PCR Primer: A Laboratory Manual, Dieffenbach and Dveksler editors, Cold Spring Harbor Press.

Companies for molecular biology products:

Promega Corporation
Invitrogen Corporation
Epicentre
USB, United States Biochemicals, now part of Affymetrix
Stratagene
New England Biolabs
Sigma Life Sciences
GE Healthcare (formerly Amersham)
Applied Biosystems