First Lab Session (Dr. Grace Jones Section of Bio 510)

Molecular cloning using pGL3 plasmid vector

I. Preparation of the vector DNA

1. Digest 10 μg plasmid DNA with 3 μl of the *Nhe* I restriction enzyme in 100 μl volume (containing 10 μl restriction buffer 2), for 3 hours (TAs will stop the reaction by performing phenol/chloroform extraction; ethanol precipitation. TAs will store it as ethanol pellet for next lab session).

II. Preparation of the insert DNA

1. Annealing the oligos. At the start of the first lab session, as the very first action to do, each group shall obtain from the TAs 8 tubes, each containing a different oligonucleotide solution (1 ug/ul). The 8 tubes correspond to 4 pairs, each pair being a tube of 'upper strand' oligonucleotide and a tube of 'lower strand' oligonucleotide. It is not necessary to kinase these oligonucleotides because they have already been synthesized to contain a 5' end phosphate group.

2. Set up annealing of each pair as follows:
   4 μg of each strand (1 $A_{260} \approx 20$ μg/ml) (concentrations of your stocks = 1μg/ml)
   20 μl 10 x annealing buffer (obtain annealing buffer from TAs)
   178 μl d H_2O
   (10 x annealing buffer: 1 M NaCl, 100 mM Tris-HCl (pH: 7.6), 10 mM EDTA)

3. Incubate the mixture at 65 °C for 5 min, and then at room temperature for 60 min.

4. During the 60 min, next cast a 2% agarose gel containing EtBR, and have it be solidifying at 4C during the 60 minute annealing incubation.

5. Remove 0.4 μg annealed double-stranded oligonucleotides (20 μl), and analyze the extent of annealing by electrophoresis using a 2% agarose gel with a 1kb ladder maker. On the gel, run one lane with 0.5 ug of one of the original single stranded oligos to mark the position of unannealed single strand. BE SURE TO USE LOADING BUFFER THAT CONTAINS A LOW AMOUNT OF BROMOPHENOL BLUE, SO THAT THE BLUE DOES NOT OBSCURE THE OLIGO BAND. This analytical 2% agarose gel will therefore contain one lane of molecular weight marker, one lane of unannealed oligomer, and four lanes, each being a sample from one of the four annealing reactions.

6. Take a picture the analytical gel result. Have the TAs verify that the annealing reaction has worked. If it has worked, proceed to the ligation step below. If it has not worked, consult the TAs about what sample the TAs have in stock that can be substituted to you so that you can continue.

7. Take the remaining 180 ul. Add 10 ul of 10x buffer that contains 10 mM ATP. Add 2 ul T4 ligase. Incubate at 16 C overnight. (TAs will stop the reaction for you the following morning by freezing you reaction tubes).

Second Lab Session of (Dr. Grace Jones Section of Bio 510)

There are five lab groups. Several persons in each group should do the section on "Preparation of the vector" while the other persons in the group do the section on "Preparation of concatamerized oligonucleotides."

Preparation of the vector

1. Obtain the vector that you left digesting from the last lab, where the TAs stopped the digestion and did phenol/chloroform and finally stored it as an ethanol pellet.
2. Remove the ethanol, briefly dry the pellet and resuspend the pellet in 50 ul of TE buffer.
3. Add 1ul of CIP enzyme to dephosphorylate the vector. Incubate at 37 C for 60 min. To inactivate the CIP enzyme, after the 60 min incubation at 1 ul of 0.5 M EDTA (pH 8.0) and heat the tube at 75 C for 10 min.
4. Spin for 30 sec after cooling to room temp. Phenol/chloroform extract.
5. Ethanol precipitate by adding 0.1 volumes of NaAcetate and 2.5 volumes of ethanol.
6. Hold as ethanol pellet at –20C until next lab meeting

Preparation of concatamerized oligonucleotides

1. Each group obtain their four tubes of concatamerized products that each group left ligating at the end of the last lab exercise (the TAs froze the ligation reactions for you at the end of the ligation period)
2. Phenol/chloroform extract.
3. Ethanol precipitate by adding 1 ul of glycogen (from TAs) and 0.1 volumes of NaAcetate and two volumes of ethanol. You ought to see a visible white pellet due to the glycogen.
4. Resuspend the ligation products in each tube in 15 ul of TE.
5. Add 1.5 ul of loading buffer.
6. In advance of the lab, the TAs will have cast four 12% acrylamide gels, one for each lab group. These gels will be used to fractionate the various sized concatamer products.
7. Each group retrieve one acrylamide gel.
8. Load the samples as follows:

Lane 1:	10 base pair marker
Lane 3:	annealed oligos for DR1A
Lane 4:	annealed oligos for DR1C
Lane 5:	annealed oligos for DR1G
Lane 6	annealed oligos for DR1T

9. Run the gel at 100 volts at room temp for approximately 60 min (or until the loading dye reaches the bottom of the gel, whichever is sooner)
10. Carefully remove the gel and visualize the bands by EtBr staining.

11. Obtain the advice of the TAs as to which bands correspond to catamers of 3 or more oligos, and using a scalpel or razor blade carefully cut out the section of the gel corresponding to these larger products.
12. Place the gel pieces into an eppendorf tube containing an “elution buffer” for eluting overnight at 37 C. (The TAs will remove the eluates into new tubes and freeze them to hold until the next lab meeting).

Third Lab Session of (Dr. Grace Jones Section of Bio 510)

There are five lab groups. Several persons in each group should do the section on "Preparation of the vector" while the other persons in the group do the section on "Preparation of concatamerized oligonucleotides."

Preparation of the vector

7. Obtain the dephosphorylated vector that you left as an ethanol pellet from the last lab.
8. Remove the ethanol, briefly dry the pellet and resuspend the pellet in 50 ul of TE buffer.
9. Estimate the amount of vector you have by running 10% of the resuspended vector on a 1% agarose gel containing EtBR. On the same gel, load a serial dilution of the 1 kb ladder. In a series of lanes, load 6 ul, 3 ul, and 1 ul of the 1 kb ladder. For the lower dilutions, you may need to dilute an aliquot of the 1 kb ladder. (E.g., to add 0.3 ul of the 1 kb ladder, take 2 ul of the ladder, and add it to 18 ul of TE, then take 3 ul to load on the gel).
10. The concentration of the lab stock of 1 kb ladder is 0.33 ug per ul. Since there are 12 bands in the ladder, then (assuming for convenience that all bands in the ladder are equal intensity – this is not true, but we will assume so for rough convenience) you can calculate the average ug of DNA in each band in the 1 kb ladder. Make this calculation for each lane of dilution of the 1 kb ladder. Now you have a rough mechanism to estimate how much DNA you have recovered from each of your four samples concatamerized products.
11. Examine the intensity of the bands in lane for each sample, and find which of the 1 kb lanes contains bands that are most similar in intensity to the sample band. If a sample band looks to be midway in intensity between two of the lanes of 1 kb bp ladder, then estimate its concentration as midway between the two.
12. Finally, convert your estimation of how much ug of concatamerized product you have to the different units of how many picomoles you have recovered for each.

Preparation of concatamerized oligonucleotides

1. Each group obtain their four tubes of frozen, eluted concatamerization products \the TAs froze the elution products for you after the last lab period.
2. Phenol/chloroform extract.
3. Ethanol precipitate by adding 0.01 volumes of glycogen (from TAs) and 0.1 volumes of NaAcetate and two volumes of ethanol. You ought to see a visible white pellet due to the glycogen.
4. Resuspend the eluted concatamerization products in each tube in 20 ul of dH20.
5. Estimate the amount of recovered products for each of the four samples (i.e., the DR1A, DR1C, DR1G and DR1T samples) by running 10% of the products of each on a 2% agarose gel containing EtBR. On the same gel, load a serial dilution of the 10

bp ladder. In a series of lanes, load 5 ul, 2 ul, and 1 ul of the 10 bp ladder. For the lower dilutions, you may need to dilute an aliquot of the 10 bp ladder. (E.g., to add 0.3 ul of the 10bp ladder, take 2 ul of the ladder, and add it to 18 ul of TE, then take 3 ul to load on the gel).

6. The concentration of the 10 bp ladder is 1 ug per ul. Since there are the equivalent of 36 bands in the ladder, then (assuming for convenience that all bands in the ladder are equal intensity) you can calculate the average ug of DNA in each band in the 10 bp ladder. Make this calculation for each lane of dilution of the 10 bp ladder. Now you have a rough mechanism to estimate how much DNA you have recovered from each of your four samples concatamerized products.
7. Examine the intensity of the bands in lane for each sample, and find which of the 10bp lanes contains bands that are most similar in intensity to the sample band. If a sample band looks to be midway in intensity between two of the lanes of 10 bp ladder, then estimate its concentration as midway between the two.
8. Finally, convert your estimation of how much ug of concatamerized product you have to the different units of how many picomoles you have recovered for each.

<u>Ligating the concatamerization products into the vector</u>.

1. For each of your four samples, set up two ligation reactions with the following ratios of DR to vector:
   a. 3 : 1 DR to vector, where the picomoles of vector is 0.03
   b. 10 : 1 DR to vector, where the picomoles of vector is 0.03

2. Set up the ligations reactions as follows:

___ ul vector
___ ul DR
___ ul water
___ ul ligase
___ ul ligation buffer (with ATP)

3. Set up the ligation reactions at 16 C for overnight. The TAs will freeze the reactions the following day.

Fourth lab exercise (Dr. Grace Jones Section Bio 510)

Cloning the reporter construct

1. Obtain from the TAs your 9 tubes of ligation products of ligating the concatamerized insert into the vector plasmid.
2. Obtain from the TAs a tube of 'competent' bacterial DH5alpha cells and keep on ice.
3. Obtain 20 ml sterile LB media without ampicillin.
4. Take 2 ul volume of you ligation mix and add to 50 ul of thawed competent cells in an eppendorf tube. Keep on ice.
5. Repeat step except use the remaining 18 ul of the ligation mix.
6. Repeat steps 4 and 5 for each of the 9 ligations that you made.
7. After 30 minutes on ice, then put the 18 eppendorf tubes into a 37 C water bath for EXACTLY 20 seconds (do not shake or mix).
8. Immediately remove the 10 eppendorf tubes back onto ice for 2 minutes.
9. Add 950 ul of LB without ampicillin to each of the 18 tubes and incubate at 37 C for 1 hr with gently shaking (225 rpm).
10. Obtain 18 LB ampicillin plates from the TAs.
11. For each of the 18 eppendorf tubes, deliver 200 ul of cells/DNA onto the plate, spread evenly by swirling and tilting the plate.
12. Cover with a lid and allow to soak into the agar in the plate at room temp on your desk.
13. The TAs later will move the plates to a 37C incubator, and after the colonies appear the TAs will move to a 4C incubator until the next lab meeting.

Mock cell transfection assay

14. During an incubation time of the above steps 1-13, coordinate with the TAs as to when your group will set up transfection of SF9 cells.
15. One group of students at a time will go to the sterile hood area an perform the following steps.
16. Seed 2 ml of cells (containing 5-7 x10^5 cells/ml (or 60%-80% confluent) per well (the medium is TC-100 medium containing antibiotics (Penicillin-Streptomycin 1%) + 10% final volume fetal bovine serum).
17. Allow cells to attach at 27^{o}C for at least one hour.
18. Prepare the following "solution-A" in sterile tubes. For each well that will receive the same transfected DNA, add to a tube the volume of DNA to be transfected (typically 0.25µg ~ 2.0µg of DNA in 1 ul, and 200µl TC-100 medium without antibiotics and without serum. For the purposes of today's mock transfection, bring with you distilled water and use distilled water instead of DNA. For the purposes of today's exercise, only one student in a group will prepare enough of solution A so that there is enough for one well of transfection per student. So, if

you have 5 students in your group, one will sit at the sterile hood and make a solution that contains 5 ul of sterile water and 500 ul of TC-100 medium without antibiotics and without serum.

19. EACH STUDENT in a group will take turns with each other student in the group so that each student ONE TIME performs the following practice exercise. Add to ONE WELL 4 ul Cellfectin Reagent that the TAs will provide at the sterile hood. Mix well by gentle swirling of the 6-well plate. When all the students have each done their practice on one of the wells in the plate, then keep the plate covered with its lid and move the plate to a place for incubation at room temperature for 40 min.
20. After the incubation, wash the cells once with 2 ml TC-100 medium without antibiotics, without serum. Do this by gently pipetting away the 2 ml that is present, and then gently adding in a new 2 ml.
21. EACH STUDENT for one well will then remove the medium in a well, and add 1.0 ml TC-100 medium without antibiotics/without serum into the washed cells. Next, add 100ul the solution-A that your group representative made earlier. Mix gently by swirling the plate.
22. Incubate cells for 5 hours in a 27^{o}C incubator.
23. The TAs will remove the transfection mixtures and add 1ml TC-100 medium containing antibiotics, and Incubate cells in a 27^{o}C until the next lab. Each of you will then examine the well you prepared to see if the cells got contaminated.

Fifth lab exercise (Dr. Grace Jones Section Bio 510)

Cloning the reporter construct

10. Obtain your platings of the bacteria transformed with the various ligation reaction products.
11. Compare the number of colonies appearing on the plates transformed with 'vector only' to those transformed with 'vector plus insert' and identify those 'vector plus insert' plates for each construct that appear to contain more colonies than the 'vector only' control.
12. Prepare a 'master plate' of LB agar onto which you will make four sets of 10 colonies.
13. Prepare 4 rows of 10 eppendorf tubes each containing 10 ul of distilled water. One will be a row of 'DR1A – 1, DR1A – 2, DR1A – 3…' and so on. The next row will DR1C – 1, DR1C – 2 .. and so on, for all four constructs.
14. For the construct of DR1A, take a toothpick and pick one of the colonies on the 'vector plus DR1A' plate and touch it lightly to the master plate, and then dip that toothpick into the tube of water that corresponds to 'DR1A-1' and shake it so that bacteria from the toothpick are shaken off into the water.
15. Take a new toothpick and touch a second colony on the 'vector plus DR1A' plate, and touch it to the second position on the 'master plate' that corresponds to position 'DR1A-2' and then dip that toothpick into the tube of water that corresponds to 'DR1A-2' and shake it so that bacteria from the toothpick are shaken off into the water.
16. Repeat step 6 for 10 colonies of DR1A, for 10 colonies of DR1C, for 10 colonies of DR1G and for 10 colonies of DR1T.
17. Each group prepare a master stock of reagents for PCR as follows:

10x PCR reaction buffer	2.5 ul
50 mM McCl2	0.75 ul
10 mM dNTP	0.5 ul
100 ng/ul primer 1	0.25 ul
100 ng/ul primer 2	0.25 ul
50 units/ul Taq DNA polymerase	0.25 ul
dHOH	18.5 ul

Multiply each of the above times the number reactions that you will perform, to calculate how much total of each reagent will go into your reaction mix. For example, if you have 40 reactions to do, then 40 x 2.5ul = 10 ul of the 10X PCR reaction buffer.

18. Prepare 40 PCR reactions using the 40 tubes that you have prepared of distilled water+bacterial colonies.

23 ul of master PCR reaction mix

2 ul of bacterial solution

19. Set up the PCR reactions with the temperature and cycling conditions of:

95 C, 1 min
95 C, 50 sec
55 C, 50 sec
72 C, 1 min

30 cycles

72 C, 10 min
4 C, indefinite

20. The TAs will come back after the lab is over and put the reaction products at 4 C until Wednesday's lab.

Cell Transfection Protocol

1. Each of the groups should examine their plate of cells to check on whether contamination got into the cells during your handling of the cells.

2. Try to again count the number of cells in a sample of SF9 cells that the TAs will have available, using the hemocytometer and the technique that we discussed in the class.

Sixth Lab Session (Dr. Grace Jones Section Bio 510)

1. Resuspend the washed bacterial pellet from the one liter bacteria culture into 20 ml of solution I.

2. Add 2 ml of a freshly prepared solution of lysozyme (10 mg/ml) in 10 mM TrisCl (pH 8.0)

3. Add 40 ml of freshly prepared solution II.

   Close the top of the centrigufe bottle and mix the contents thoroughly by gently inverting the bottle several times. Store the bottle at room temperature for 5-10 minutes.

4. Add 20 ml of ice-cold solution III.

   Close the top of the centrifuge bottle and mix the contents by shaking the bottle several times. There should no longer be two distinguishable liquid phases. Store the bottle on ice for 10 minutes. A flocculent white precipitate should form.

5. Centrifuge the bacterial lysate at 4000 rpm for 15 min at 4 C in the equivalent of a Sorvall Gs3 rotor. Allow the rotor to stop without using the brake.

6. Filter the supernatant through four layers of cheesecloth into a 250 ml centrifuge bottle. Add 0.6 volumes of isopropanol, mix well, and store the bottle for 10 minutes at room temperature.

7. Recover the nucleic acids by centrifugation at 5000 rpm for 15 minutes at room temperature.

8. Decant the supernatant carefully, and invert the open bottle to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the bottle with 70% ethanol at room temperature. Drain off the ethanol, and use a pasteur pipette attached to a vacuum line to remove any beads of liquid that adhere to the walls of the bottle. Place the inverted, open bottle on a pad of paper towels for a few minutes at room temperature to allow the final trace of ethanol to evaporate.

9. Dissolve the pellet of nucleic acids in 8 ml of TE (pH 8.0).

10. Purify the DNA by CsCl ethidium bromide gradient centrifugation. (The DNA will be placed onto the CsCl for the students, and centrifugation started. Students at next lab meeting will stop the centrifugation and recover the DNA)
    (adapted from Sambrook et al., pp 138,1.39)

Seventh Lab Session (Dr. Grace Jones Section, Bio 510)

1. Remove the ultracentrifuge tubes containing the DNA samples, that have been spinning for 30 hr in the ultracentrifuge. Each group has one tube.
2. Bring the tube to you desk. Put the tube into a clamp that is held above the surface of the lab bench by a ring stand. Be sure that a bench top 'diaper' is underneath the stand.
3. As in the diagram attached (from Sambrook et al), the band that corresponds to the DNA sample must be removed with a syringe needle (CAREFUL NOT TO JAB YOURSELF WITH THE NEEDLE!!!!). A TA will come to you lab bench and confirm that band of interest, and that TA will puncture the tube with the syringe needle and start to withdraw the sample.
4. A student from the group will then take over the sample and complete the withdrawing of the DNA band.
5. Gently squirt the DNA sample from the syringe into one to several eppendorf tubes (depending on volume). Put about one half ml of the DNA sample into each eppendorf tube.
6. Add an equal volume of isopropanol that is saturated with CsCl. This will form a separate phase above the aqueous CsCl/DNA sample.
7. Using a one ml pipetteman, draw both phases up and down into the pipette tip, until the two phases have been well mixed. The EtBr (red in color) will partition into the isopropanol phase.
8. Spin the eppendorf tubes briefly (say, 1 min) to cleanly separate the two phases. Using a one ml pipetteman, reach down through the isopropanol phase and withdraw the aqueous DNA sample of interest. (Provide to the TAs the remaining isopropanol-containing eppendorf tubes and the ultracentrifuge tube containing the CsCl gradient containing EtBr so that the TA will discard of it properly).
9. Repeat steps 6-8 until all the pink color is gone from both phases.
10. Now, remove the CsCl from the DNA sample by dialysis. The TA has prepared in advance for you the dialysis tubing. For each eppedorf tube that you have containing DNA sample, you will prepare a 'dialysis bag' of a strip of the dialysis tubing that is clamped tightly in each end with a special dialysis tubing clamp. The strip of dialysis tubing is cut to be about 2 inches long. Toward one end, put one clamp. Then, use the one ml pipetteman to transfer the DNA sample into the dialysis tubing through the still single open end of the tubing. Then, place the second clamp over the remaining open end, but try to squeeze out as a much as possible any air from the DNA sample that is trapped between the two clamps (one clamp still loose while you are doing this). A TA will demonstrate this procedure on the first sample from your first eppendorf tube. Then, the students in the group will prepare the DNA samples in their remaining eppendorf tubes themselves.
11. As you make them, place the dialysis bag samples into a 1 liter beaker that is full of distilled water.
12. This beaker will be placed at 4 C until the next lab. The TAs will make one or two changes of the distilled water during that time.
13. At the next lab session, you will recover the dialyzed sample, precipitate it, quantify it, and prepare it for cell transfection.

Eighth Lab Session (Dr. Grace Jones Section Bio 510)

1. Lepidopteran SF9 cells, derived from *Spodoptera frugiperda* (Noctuidae), are being cultured at 25°C in TC100 media (Gibco-BRL).

2. For transfection, plate approximately 10^6 cell/ml into 6-well Corning plastic trays, and allowed to attached to the well surface for at least 1 hr (TA will do this ahead of the lab start time).

3. Each group prepare the following 5 constructs for transfection:

    a. pGL3 vector only

    b. JHECore pGL3

    c. DR1(two)JHECore pGL3

    d. DR1(five – F) JHECore pGL3

    e. DR1(five – C) JHECore pGL3

    Obtain the DNA for a, b, c, and d from the TAs. The fifth DNA is the DNA construct that your group made in the class.

4 UNDER STERILE CONDITIONS IN A CELL CULTURE HOOD. In an eppendorf tube, add 1.5 ug of pGL3 vector only DNA, and add to it 24 ul of Cellfectin plus 100 of TC100 medium that is without serum ("incomplete" medium). (This is a 6x preparation, since you will divide this into three replicate wells of cells later).

5 Repeat step 4 for each of the five constructs.

6 Allow the mixes of each of the five constructs to incubate with the Cellfectin for 40 min. (From our class lecture, what is happening during this incubation). The next group will then come to the hood to do this part, etc., and so on for all four lab groups.

7 When the incubation time is complete for the first group, the first group will return to the sterile culture hood and bring into the hood the five 6-well plates into which SF9 cells were previously placed for them by TA. Plan and mark on the lids of the five plates which well is to get which DNA. Remember each DNA will be replicated into three wells. So, the first plate has six wells, three for one DNA, three for another. And so on for all five plates and all five DNA constructs.

8 Open the lid on the first plate and obtain the eppendorf tube of DNA plus Cellfectin that corresponds to the DNA that will go into the first six

wells. Take one third of the volume of DNA plus Cellfectin and add it to the first well. And so on for the remaining volume.

9 Next, obtain the eppendorf tube that corresponds to the next DNA, and repeat step 8 for that DNA.

10 Close the lid of that plate and set that plate into the controlled incubation chamber.

11 Next, obtain the second plate of cells that was prepared in advance for you, and perform steps 8, 9 and 10 for the next two DNA constructs.

12 Finally, perform steps 8, 9 and 10 for the remaining, fifth, DNA construct.

13 When you have completed the initiation of the transfection protocol, all five of your plates of transfected cells will be back in the controlled temperature incubator.

14 What SHOULD happen at this point, is that between 5 hrs to overnight later, you return and remove the medium/DNA/Cellfectin from each well, and replace it with 1 ml of incomplete medium, and then add a final 2 ml of fresh medium to each well and replace the cells into the incubator. Then, you would come back one or two days later and add the test hormones to each well.

15 What will happen instead is that a TA will kindly come for you tomorrow morning and perform the washing and hormone addition steps. Therefore, the experimental cells will be ready and waiting for you at the next lab meeting for you to harvest and analyze for expression of the reporter gene.

Ninth Lab Session (Dr. Grace Jones Section Bio 510)

1. During the time since the last lab session, a TA has both stopped the transfection process, and then also added 24 hr ago either the ecdysone hormone (a steroid hormone) or only the EtOH control (which is what the ecdysone is dissolved in).
2. Each group will obtain the plastic 6-well plates that contain their transfection reactions. (Sterile are not needed beginning at this point). Open the lids and for each well, use a one ml pipetteman to gently draw up one ml of the two ml of medium, then gently squirt the medium back down onto the cells in the well to dislodge them from adhering to the bottom of the well. Do this several times until all of the cells have been dislodged. Be careful not to do this so vigorously that the cells break. Each student can be doing this on one of the plates of their group, so that you can get all the plates of your group done quickly.
3. Set up a row of microfuge tubes, in which each tube is labeled to correspond one of the wells of your cell transfection.
4. For each well, agitate the now loose cells in the well so that the cells are at least briefly floating around in a uniform suspension. Quickly draw one ml of cells from the well and put it into an microfuge tube labeled for that sample. You must do this quickly before the cells start to settle and the suspension becomes not uniform.
5. When all the 1 ml samples are transferred to their respective microfuge tubes, spin them briefly for one minute in a microfuge, just to pellet the cells (but we don't want them to break).
6. Remove and discard the supernatant.
7. Add to each tube 400 microliters of lysis buffer. Mix well by drawing the pellet and lysis buffer up and down several times into a one ml pipetteman, to lyse the cells.
8. Obtain pieces of dry ice into a dry ice bucket, and quickly freeze the tubes containing the lysate. (If dry ice is not available, then put the samples into a –80C freezer to freeze them).
9. After 15 minutes of freezing, thaw back out the lysate of one of the tubes for a sample that should contain very high activity (that is, the treatments that received a construct with 5 copies of the DR and that were treated with ecdysone likely have the highest activity. So, select one of those tubes to thaw back out). The remaining samples should stay frozen at –80C until the next lab meeting. The TAs will arrange to keep them frozen at –80C.
10. For the single tube that you have thawed back out and are keeping on wet ice, prepare 4 microfuge tubes as follows:

____ ul lysate
100 ul luciferase substrate (the substrate will be prepared by a TA)

Make a range of lysate to be added as 0 ul, 5 ul, 10 ul, 20 ul. So, you will have four tubes of assay that you will need to count in the scintillation counter according to the procedure you were shown last week.

11. So, get everything ready, that the scintillation counter is available for you, and then add the lysate to the luciferase substrate, and briefly vortex for a few seconds, and then measure the light output in the scintillation counter. Try to practice setting up a staggered system in which you start each of you reactions at 30 seconds apart and that they are measured in the scintillation counter approximately 30 seconds apart.
12. Analyze data you obtain as a graphical plot of cpm vs. lysate amount, and observe what is the largest amount of lysate that you can add and the cpm obtained is still on the linear part of the plot. On the next lab, you will then assay all remaining samples using that amount of lysate.

Tenth Lab Exercise (Dr. Grace Jones Section Bio 510)

1. Today, you will perform the luciferase assay on the remaining 29 (of 30) samples from your experiment.
2. Normally, you would perform the experiment in small subsets, so that the short time intervals between measurement of each vial in the scintillation counter can be maintained. However, because we have four groups all needing prompt access to the scintillation counter once the assay has started, we will for the purpose of this lab not consider the need to keep the time intervals short and consistent.
3. The TAs will designate the lab groups as "first", "second", "third" and "fourth." The first group will thaw out the 29 microfuge tubes of cell lysate extracts, and allow them to warm to room temperature (the TAs might do this in advance of the lab meeting).
4. Prepare a second series of 29 "assay" microfuge tubes, each corresponding to one of the 29 "lysate" tubes.
5. Utilize the data from the last lab meeting to determine the maximum amount of cell lysate (5, 10 or 20 ul) that you can use and still be in the linear region of the assay kinetics.
6. Aliquot that amount of each of the 29 "lysate" samples into the respective "assay" tubes. (Someone in the group be getting 29 scintillation vials into scintillation counter racks).
7. Make sure at this point that the scintillation counter is not being used and is ready to receive your tubes. To each assay tube, add 100 microliters of luciferase substrate (try to do this as quickly as possible). Shake each tube briefly to mix each sample.
8. Quickly place the 29 assay microfuge tubes into 29 scintillation vials, as you did at the last lab meeting, and quickly put the 29 vials into scintillation racks and start counting them in the scintillation counter.
9. When the scintillation counting of the first group is 5-10 minutes away from finishing, the TAs (who will be closely monitoring the progress of the scintillation counting) will alert the second group to perform steps 3-8. And so on later for groups 3 and 4.
10. Analyze the resulting data to determine the average ($\pm$ standard deviation) of the three replications you made of each experimental treatment (you had 10 experimental treatments – what were they?).
11. Analyze the results to determine whether increasing the number of DR elements changed the activity (a) in the absence of hormone or (b) in the presence of hormone. That is, compare the activity of the different DR number constructs for the case where each received only ethanol. Then, compare the ratio of the ecdysone treatment/ethanol treatment for each DR construct. Is the ratio significantly different for the situation of two DR elements vs. five DR elements?
12. Perform the same kind of analysis as above in item 11, except this time the question you are considering is whether the differences in orientations, when the DR number is the same (we have two constructs each with five DRs), has any effect on (a) the level of activity in the absence of hormone or (b) on the ratio of activities of the ecdysone treatment/ethanol treatment.