# pGLO MUTAGENESIS SESSION FIVE

## Procedures

Each table can be divided into 3 groups; one group will work on A- mutant one on G- mutant and the third on pGLO controls. The whole table will then make a gel and load their samples.

**Restriction Digestion** (Also See Appenix at the end of the Lab Manual)

Our next step is to digest the plasmid DNAs that we isolated in the previous session. We will use the combination of Nde I and Psi I. This is only one set of restriction enzymes (REs) that produces adequate numbers of fragments with good separation and uses the same buffer system for their activity. There may be many other RE combinations that one could use for the same objective. Based on the size of the cut fragments as compared to the control (pGLO plasmid), we can determine which fragment is carrying the Tn transposon.

## Laboratory Supplies

| | |
|---|---|
| Eppendorf tubes, 0.5 ml capacity | 1 beaker/table |
| Loading dye | 1vial/table |
| pGLO, 50 ng/$\mu$l | 5 $\mu$l/group |
| Sterile water | 1vial/table |
| Nde I & Psi I | 1 vial of each/table |
| Psi I | 1 vial/table |
| NEBuffer 4 | 1 vial/table |
| Water bath (37°C), Heat block (70°C) | 1 of each/table |

## Procedures

1. Get ice and label 3 sets of 2 0.5 ml Eppendorf tubes. Thaw out your best A and G mutant DNAs based on electrophoretic properties in the previous session. For each set, you want to have the uncut plasmid and the cut plasmid. You can use the following table as a guideline for control ($A^RK^SG^+$ = pGLO plasmid) and two types of mutants $A^SK^RG^+$ and $A^RK^RG^-$. In each case, use 2.5 $\mu$l of your plasmid prep DNA, 1 $\mu$l of the buffer and 1 $\mu$l of each RE. Add water to bring the total volume to 10 $\mu$l. Keep tubes on ice.

| Tube num | Tubes | Genotype | DNA | Water | NEBuffer 4 | Nde I | Psi I |
|---|---|---|---|---|---|---|---|
| 2 | Uncut pGLO | $A^RK^SG^+$ | 2.5 $\mu$l | 7.5 $\mu$l | - | - | - |
| 3 | Uncut $A^-$ | $A^SK^RG^+$ | 2.5 $\mu$l | 7.5 $\mu$l | - | - | - |
| 4 | Uncut $G^-$ | $A^RK^RG^-$ | 2.5 $\mu$l | 7.5 $\mu$l | - | - | - |
| 5 | Cut pGLO | $A^RK^SG^+$ | 2.5 $\mu$l | 4.5 $\mu$l | 1 $\mu$l | 1 $\mu$l | 1 $\mu$l |
| 6 | Cut $A^-$ | $A^SK^RG^+$ | 2.5 $\mu$l | 4.5 $\mu$l | 1 $\mu$l | 1 $\mu$l | 1 $\mu$l |
| 7 | Cut $G^-$ | $A^RK^RG^-$ | 2.5 $\mu$l | 4.5 $\mu$l | 1 $\mu$l | 1 $\mu$l | 1 $\mu$l |



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Note 1: If you failed to obtain concentrated, clean bands on your gel in the previous session, ask your TA to get you DNA from a group that did.

Note 2: Tube number 1 is reserved for the standard (see step 1 of the next procedure).

Note 3: Make sure you change pipet tips when going from one vial to the next.

2. Vortex tubes well and spin for a few seconds and incubate tubes 5, 6 and 7 at 37°C for 1 hour. At 15-minute intervals, vortex and spin down these tubes and return them to 37°C. Keep the rest of tubes on ice.

**Transposon Insertion Mapping**

## Laboratory Supplies

| | |
|---|---|
| Standard DNA (50 ng/$\mu$l) | 1 tube/lab |
| Eppendorf tubes | 1 beaker/table |
| Microfuge | 2/lab |
| Loading dye | 1 tube/lab |
| Bio-Rad gel apparatus + 8-tooth comb | 1/table |
| Sterile distilled water | 1 tube/table |
| Power supply | 1/table |
| Agarose | 1 bottle/table |
| Balance | 1/lab |
| Heat-resistant gloves | 1 pair/lab |
| Electrophoresis buffer, TAE (1X) | 5 L/lab |
| Flask, 125 ml | 1/group |
| Spatula | 1/lab |
| Graduated cylinder, 100 ml | 2/lab |
| Graduated cylinder, 1 L | 2/lab |
| Microwave oven | 1/lab |
| SYBR Gold | 1 tube/lab |
| Tape, roll | 1/table |
| Photography equipment | 1/lab |
| Pipetman, one set of P20 and P200 | 1/group |
| Pipet tips, large and small | 1 box of each/group |

## Procedures

1. Prepare a tube of 1 Kb standard by adding 100 ng of the DNA ladder and bringing it up to 10 $\mu$l with sterile water. Label this as Tube #1.

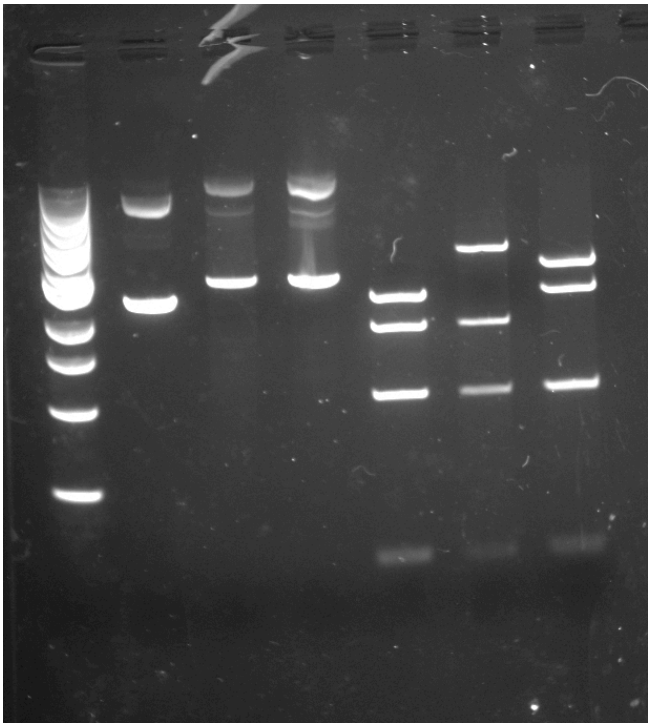
2. Add 2 $\mu$l of loading dye to all the tubes, mix and spin for a few seconds to bring all liquids to the bottom of tubes. Leave tubes on ice until needed.

3. Obtain a gel apparatus and prepare a gel as described before. Load your samples and run the gel at 100 volts and 400 milliamperes for 60 minutes.

4. Check the leading dye movement to make sure that everything is going fine. If you do not see any dye color appearing within 5-10 minutes, check to make sure your connections are secure and correctly connected. When the run is complete, take a photograph of the gel.

   Note: While the gel is running, prepare your samples for sequencing (see the next part).

Figure: A group's gel photograph: From left to right, lanes 1 to 7 are respectively loaded with standard, uncut pGLO, uncut A-, uncut G-, cut pGLO, cut A- and cut G- (See table on previous section)



**Sequencing pGLO From Each Target Site**

We get some idea of the location of the transposon insertion into pGLO based on restriction digestion analyses. However, to learn the exact location of the insertion, we need to sequence the DNA at the two sides of where Tn has lodged itself. For this purpose, we use the forward and reverse primers that have exact homology to the nucleotides near the two ends of the transposon (adjacent to MEs). When we look at sequencing results, we should look beyond the Mosaic End sequences because it is after these sequences that the pGLO sequence starts. Thus, the ME is a good clue that can show the juncture region of transposon and pGLO. As mentioned before, the complete sequence of EZ-Tn5 can be obtained from Epicentre's Website (http://www.epibio.com/sequences/Kan2.asp).

## Laboratory Supplies

| | |
|---|---|
| KAN-2 FP-1 Forward Primer (1/40 dilution) | 3 $\mu$l/sample |
| KAN-2 RP-1 Reverse Primer (1/40 dilution) | 3 $\mu$l/sample |
| Small Eppendorfs, 0.5 ml capacity | 1 beaker/table |
| Tiny Eppendorfs (0.2 ml capacity) | as many sets of 8 as needed |
| Tiny Eppendorf rack | 1/table |
| Sequencing Form | 1/lab |

## Procedures

1. Thaw the original plasmid mini-prep tubes (the one best A and one best G mutant that you chose in "pM: Session 4" based on electrophoretic properties) on ice.

2. Obtain 4 small Eppendorf tubes (0.5 ml capacity) and label them AF, AR, GF and GR. A and G stand for Amp and GFP mutants and F and R denote the forward and reverse primers. Add 7 $\mu$l of each of your A plasmid to AF and AR tubes and 7 $\mu$l of your G plasmid to GF and GR tubes. Now to tubes having the designation F, add 3 $\mu$l of the forward primer and to the other tubes, add 3 $\mu$l of the reverse primer (1/40 dilutions). Add your initial to the tube labels.

3. Mix the contents well and spin for a few seconds and take your tubes to your TA so he/she can fill out a Sequencing Form. Write down the labels of your samples in your notebook, as the names of the sequence files returned from Penn Sequencing Facilities (UPSF) would be the same as these labels. Your TA needs to transfer your samples into tiny Eppendorf tubes (0.2 ml capacity) required by UPSF for automated processing.

   Note to TA: The format of the labels for samples being sent to UPSF should be: SSS-(AR, AF, GR or GF)-T# where SSS is your section number and # is the table number in the lab. For example the samples of students at Table 1 of section 101 should be labeled as:
   101-AR-T1
   101-AF-T1
   101-GR-T1
   101-GF-T1

### Determining the Site of Transposon Insertion

1. Shortly, we will have the sequencing results and the files will be posted on the course BlackBoard. You need to go to that site and download your sequence files to your computer.

   Note: We will upload all the sequencing files returned to us from UPSF on the course BlackBoard regardless of their content. You can find these files in "Laboratory Documents" under "Transposon Sequencing Results" under "Students' Results". If you find that your group's files are not usable or are giving unexpected results, you need to write your report based on one of the sequences that we place in a folder named "Replacement Sequences". Use the last digit of your Penn ID to determine which file sets to use (for example, if your Penn ID ends with 3, you have to use the file set #3).



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2. Open your browser (Netscape, Internet Explorer, etc.) and go to the ncbi site (http://www.ncbi.nlm.nih.gov). In the search box, change the drop down menu from "All Databases" to "Nucleotide" and in the lower box, type the actual name of the pGLO plasmid, namely "pBAD-GFPuv" and then click on "Search".

3. A new page will appear that contains information about the sequence. Look through the data carefully and especially note the size of the plasmid, the genes present, the location of each gene on the plasmid and the sequence of bases. For example, note that the gene *AraC* occupies the base positions 96 to 974. Draw the plasmid and place all the markers as well as the number of base sequences for start and end of each gene. Label your drawing with all the other data that you think are important. If you know how to use software such as MacVector, Vector NTI, DNASTAR Lasergene, etc, you may use them to obtain a concise map of the plasmid with color notations and markers. We encourage you to draw the map of pGLO yourself but you can also use the map given in the introduction of this topic or from elsewhere.

4. You now need to copy the base sequences of pGLO in a text format either from the ncbi site or other sites onto your computer. Simply go to top of the page and click on the FASTA format. Now you can copy the pure nucleotide sequence (i.e., without the introduction line) and paste it into a text file on your desktop.

5. Use your browser to go to the original ncbi site once more and click on BLAST. You have already worked with BLAST (Basic Local Alignment Search Tool). However, here we are using a special type of blast called "bl2seq". It is found under Specialized BLAST "Align two sequences using BLAST (bl2seq)" on the BLAST page. "bl2seq" can be given any two sequences and it will try to align the homologous areas.

6. Click on "Align" to open up the main bl2seq page. Then paste pGLO DNA sequence in the "Sequence 1" box and AF sequence in the "Sequence 2" box and click on the "BLAST" button at the bottom of the page. Leave all parameters at their default values. Print the outcome and then clear Sequence 2 box and paste your AR sequence. Again print the results. For our data we want to compare the forward and reverse sequences of each of our plasmids to the pGLO sequence to see where the homology is in each case and from that, deduce where the point of Tn insertion is. Note that Query is the sequence of pBAD and Sbjct is our sample's sequence and so we should note down the Query sequence number for comparison and not the Sbjct's. For example, if after using bl2seq, we find that the forward primer has produced a homology with Query base numbers 873 to 1800 and the reverse primer has produced a homology with Query base numbers 2746 to 1792, then we can deduce that the transposon has inserted itself into pGLO approximately at base positions 1792-1800. Next we need to look at our plasmid map to see which gene is involved at this location (see step 3 above) and if that is consistent with our expectations.

   Note 1: Make sure you check %Identities on the alignment page. It should be close to 100%, otherwise, you need to repeat the sequencing.

   Note 2: Disregard short alignments (e.g. 2089-2119 or 2324-2367) that may be due to some affinity to the polycloning site of pGLO. Consider only the longest sequence homology.

Sometimes there may be two large sequence homologies due to the fact that pGLO is circular but alignment is done in a linear fashion (i.e., position “0” of pGLO falls somewhere in between the sequence).

Note 3: Use the map of pGLO you prepared to aid you in deciding the site of incorporation of the transposon.

**Pinpointing the Site of Transposon Insertion**

Undoubtedly you have looked at the alignment of the forward (F) and reverse (R) sequences of your samples sent to UPSF and have found that there is some overlap of nucleotides. At this point we are able to exactly pinpoint the point of insertion of the transposon into our target DNA (pGLO). However, knowing that the transposon carries a 19 base repeat (known as ME sequences) at each side makes our job of determining where it is inserted easier. This 19 bp can act as the recognition site at the junction of the transposon and the target DNA. So one easy way is to look for this sequence in our sequencing file and then deleting this sequence as well as all the bases before it. We can actually use the "Find" menu option of a Word processor application with the ME sequence in the “Find” box for this purpose. After finding and deleting all unwanted bases, one needs to resave the document as text and do the realignment with bl2seq as was described previously.

Note 1: Some sequences do not contain the ME nucleotides.

Note 2: Sometimes cleaning up of the DNA from these extra bases does not improve the conclusions drawn.

Note 3: In some cases, it may be easier to visually look at the sequencing data and find the 19 bp ME (AGATGTGTATAAGAGACAG) because the “Find” function of a word processor would not be able to find the sequence if even a single base is different in your file. If you are using this option, look at the first 50-100 bases of your sequence and you should be able to determine which bases to delete.

The insertion of transposon into a target DNA can be likened to that of the staggered recognition site of many restriction enzymes. That means that the Tn5 transposase does not cut the two strands of the target DNA bluntly; it rather produces staggered target DNA of 9 bp. After the transposon has inserted itself into the cut, the staggered single strands are duplicated by complementarity via transposase. This is the reason for the presence of a 9 bp repeat flanking a transposon insertion site. Of course, the sequences of these 9 bases are different in different clones, because the insertion happens at random.

## Results of pGLO Mutagenesis Lab Exercise
## pM: Session Five

Name _____ Date _____ Section _____

Name of partner(s) _____

Write a short typed paper. This paper, like the one on PCR-ID, should include a Cover Sheet, Introduction, Materials and Methods and Results and Discussions. Submit a copy of your report to the BlackBoard. Use TN instead of EK or ID in the name of your file (i.e., 123sssTN-TYY-XxxxxxZ.doc). The following points should be included in your paper:

1. Find an application or program on the Internet that could show you what fragments are obtained when a piece of DNA is cut by one or more particular restriction enzymes and justify the use of Nde I and Psi I that you used on pGLO DNA. [Hint: you may obtain the sequence of pGLO DNA as mentioned earlier and use NEBCutter2 from http://tools.neb.com/NEBcutter2/index.php].

2. Include a copy of your gel photograph and explain the bands that you observed. Do the positions of the bands agree with your expectation? Discuss. Can you locate the band(s) that is/are carrying the transposon?

3. Can you suggest another set of restriction enzymes that may give us as good or better results as the set you used in the lab? Discuss.

4. Align the nucleotide sequence of pGLO with each of AF, AR, GF and GR sequences using bl2seq and include the printouts of your results with this report. Do the alignments of the pGLO sequence with AF, AR, GF and GR sequences agree with your expectations? Explain.

5. Draw the map of pGLO with all the genes and label with restriction sites for Nde I and Psi I as well as the REs you found in Question 3 above. Show the alignments of AF, AR, GF and GR sequences on this same map.

6. Try bl2seq to see if there are any homologies between your plasmid's sequence (each of your AF, AR, GF and GR sequences) as well as the mosaic ends (MEs). Discuss your findings and explain whether they are in accordance with your expectations.

7. Pinpoint the insertion point of the Tn5 into pGLO for your mutants (before and after excluding MEs) and discuss your general conclusions.



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