

HUMAN CDK3 SNP LAB I. DNA ISOLATION AND PCR

Special thanks to Julia VanderMeer, Lydia McClure, and Heidi Mullen for their work developing the lab protocol.

Project Goals

For the first part of this two-week lab project, you will isolate your own DNA from cheek cells and set up a PCR reaction. The PCR will amplify a region of DNA which contains a SNP; the SNP we are investigating is from an intron in the Cdk3 gene. Next week, you will attempt to digest your PCR product with a restriction enzyme which recognizes one variant of this SNP but not the other variant. You will gain an understanding of using PCR to amplify a specific segment of DNA and using RFLP to detect different forms of a SNP.

The Cdk3 SNP

SNPs are single nucleotide polymorphisms; they represent the simplest type of genetic variation between individuals. A SNP refers to a specific location in the genome where different people have been shown to have a different nucleotide. For example, the SNP we will begin investigating in lab this week is on chromosome 17, and it is located at base pair 71,511,227 (chromosome 17 has approximately 80,000,000 base pairs). Some people have an “A” in this position, and other people have a “G” in this position.

If more than one percent of the population has a different base at a particular location, that location is considered a SNP. If less than one percent of the population has a different base, it is considered a mutation.

The SNP we are investigating is located in intron 6 of the Cdk3 gene. The Cdk3 protein is one specific type of Cdk (cyclin dependent kinase); Cdks help regulate a cell's progression through the phases of the cell cycle. Cdk function is dependent on binding to other proteins called cyclins. Cdk3 is one of the Cdks involved in allowing the cell to move into the S phase of the cell cycle.

The particular polymorphism we are investigating is highly unlikely to have any phenotypic effect (why?). We will refer to the two different variations at this position as the “A allele” and the “G allele.”

Testing for the Cdk3 SNP

You will actually be testing your own DNA to see what your genotype at this SNP is. There will be four major steps in this project:

1. Isolate DNA from your cells.
2. Amplify the region of DNA containing the SNP.
3. Digest the DNA with a restriction enzyme.
4. Run the product(s) of the digest on a gel.

Today, you will perform the first two parts of the project; next week, you will complete the project.

DNA Isolation

You will use a kit to isolate your DNA. You will begin with cells you have scraped (gently!) from the inside of your cheeks. You will break these cells open (chemically) and use a spin column to trap the DNA from the cells.

Amplification of the SNP Region Using PCR

Next, you will set up a PCR reaction designed to amplify the region of DNA surrounding the SNP (Fig. 1). We need to use PCR so we will have many copies of the particular DNA we are interested in; we do not want to try to experiment with the entire chromosome (remember how big it was!). We need many copies in order to be able to visualize the DNA on a gel.

If we tried to load the entire genomic DNA on a gel, it would mostly be stuck in the well; chunks of DNA which had been inadvertently cut during the DNA isolation procedure might move through the gel and create a smear in the lane, but it would be quite difficult to interpret the gel.

5' AAGGGCGTGT AGCACAGCAT AAAGACAGAG CTAACCTCAAT GAGCGCCACT TTCACAGGGA
 AGATAAATAC TGCACCTTATC CTGGGGGAGG CTTCC^AGGTT GAACAATCAG TATACCCAAG
 CCAGTTGTGT ACAAAGGTCA GGAAAGAGAC CCTGGCCTTG GACTCAGAAA GTGCCAGGGT
 TATGTAAGAG GCTGGCTGAT GAGGGGAAAC TGTAGTCGGA GCAGCAGCTG GAGCCCACAT
 GCACCTACCA TGAGCAGGTC CCTGCCCTCT GGCTCCAGAT TGGGCACAAT CTCTTCCAGT
 CCCTTCCTGG T 3'

Figure 1. The DNA Sequence we will amplify with PCR. Note that only one strand of DNA is shown. The SNP is indicated in a box; it is denoted as an "A" but could also be a "G." The spaces have been inserted only to make counting bases simpler; they have no function in the sequence other than to show the bases in groups of ten.

We will use primers which are 24 base pairs long. Using what you know about PCR, what primers will we use to amplify this region of DNA? Write the sequence in the box below; indicate the 5' and 3' ends.

Primers for PCR:

| Allele | Hpa II cut? | Fragment Length(s) |
|--------|-------------|--------------------|
| A | | |
| G | | |

Running the Digest Products on a Gel

By now you should have some idea of how a gel will help you determine your genotype with regard to this SNP. In the space below, draw a picture of a gel with three lanes: one lane for someone homozygous for the "A" allele, a "G" homozygote, and a heterozygote. Indicate (in base pairs) what size you expect each band to represent.

DNA Digestion with the Restriction Enzyme Hpa II

Next week, once you have plenty of copies of the region of DNA we're interested in, you will set up a restriction enzyme digest. How will this tell you what SNP you have? Restriction enzymes are quite specific about what DNA sequences they will cut. If the sequence is different, *even by one base*, then they will not cut. We have made use of this fact by choosing a restriction enzyme which will cut one variety of the SNP, but not the other.

The site which the restriction enzyme Hpa II recognizes is 5' CCGG 3'. Hpa II cuts the sugar-phosphate backbone between the two C's at the 5' end of the sequence: 5' C|CGG 3'. Using the position of the SNP in the sequence above, which allele will be cut by Hpa II? How many base pairs will the resulting DNA fragments be? Write your answers below.

This general type of analysis is called "RFLP," which stands for Restriction Fragment Length Polymorphism. In RFLP analysis, you cut DNA with restriction enzymes, determine the sizes of the resulting DNA fragments using a gel, and infer something about the original DNA based on the lengths of these fragments. In our case, we can determine the allele of our SNP just by looking at the size of the fragments after cutting.

Experimental Procedures:

You should wear gloves for the remainder of the procedures in lab today, in order to prevent contamination of your DNA sample. In addition, you should only work with your own DNA sample, not your lab partners' samples.

Cheek Cell Collection

1. Scrape a sterile swab against the inside of each cheek for approximately 20 seconds. Air-dry the swab for 15 minutes after collection, by placing the handle of the swab in a 15-ml plastic, conical tube at your bench. **Do not consume food or drink for 30 minutes before collection.**

PBS is phosphate-buffered saline.

3. When the swab has dried for 15 minutes, eject the tip containing your cheek cells into the PBS in the microfuge tube. To eject the tip, press the stem end toward the swab.

DNA Isolation

4. Add 20 µl of proteinase K to the microfuge tube.

Your lab instructor or TA will have the tube of Proteinase K. Take your tube to them for pipetting. Proteinase K is an enzyme which lyses (breaks open) the cells and breaks down proteins. Some of the proteins broken down by proteinase K include enzymes which might otherwise break down your DNA.

5. Add 400 µl Buffer AL and immediately mix by vortexing for 15 seconds. Make sure the lid on your tube is tightly closed before vortexing.

This buffer contains a high salt concentration, which will help your DNA bind to the silica-gel-membrane of the spin column (below).

6. Incubate your tube at 56°C for 10 minutes.

This incubation is to give the cells time to lyse.

7. Microfuge your tube briefly (around 15 seconds) to remove any drops of condensation from inside the lid. (Don't forget to balance your tube with someone else's tube.)

8. Add 400 µl of ethanol to the microfuge tube. Mix thoroughly by vortexing for 15 seconds. Briefly

microfuge to prevent your solution from sticking to the inside of the lid.

9. Locate a DNeasy spin column and three collection tubes. Remove 700µl of your sample and apply to the DNeasy spin column (while the column is in one of the collection tubes). Microfuge at 8000 rpm for 1 minute.

These spin columns will only hold about 700 µl of fluid, so you will repeat this process in step 11 in order to move all your DNA onto a single spin column.

10. Remove the spin column and discard the flow-through by dumping the collection tube into your liquid waste beaker.

11. Place the spin column back into the same collection tube (which should now be empty). Add the remainder of your sample to the spin column. Microfuge at 8000 rpm for 1 minute.

If you have any precipitate which has formed in your original tube by this stage, pipette all of this (along with the liquid) onto the spin column. The DNA from your cells is now binding to the silica-gel-membrane in the spin column.

12. Remove the spin column and discard both the flow-through and the collection tube into the appropriate waste beakers. Place the spin column in a new, clean collection tube.

13. Open the spin column and add 500 µl of Buffer AW1. Microfuge at 8000 rpm for 1 minute.

14. Discard the collection tube and the flow-through. Place the column in a new collection tube.

15. Open the spin column and add 500 µl of Buffer AW2. Close the cap and microfuge at maximum speed for 3 minutes. When removing your tube from the microfuge, be careful not to let the bottom of the spin column come into contact with the flow-through.

16. Remove the spin column from the collection tube and place the spin column in a labeled microfuge tube. Discard the collection tube.

17. Add 150 µl Buffer AE to the spin column. Let the column sit at room temperature for 1 minute. Then, microfuge for 1 minute at 8000 rpm. When you microfuge, the cap of your tube will be open; check the direction the microfuge rotor goes in, and make sure your cap is trailing the spin column and tube (ask your lab instructor or TA if you

have questions). The DNA will be eluted (pulled off) the membrane and be in the microfuge tube.

Buffer AE has a very low salt concentration, which helps pull the DNA off the silica-gel-membrane of the spin column. Congratulations! You have now isolated your own DNA into a microfuge tube!

18. Store your DNA on ice until you are ready to set up the PCR reaction.

Prepare Your DNA for PCR

19. Locate a PCR tube containing a master mix bead.

Marvel at the size of this tube. "Master mix" is the commonly used term for this subset of the PCR components: it contains TAQ polymerase, dNTPs, and buffer. In this case, the components have been dried into a bead, so you'll dissolve the bead into solution using your primers and DNA.

20. Pipette 24 μ l of primers into your PCR tube.

This tube contains a combination of the two primers necessary to replicate both strands of your DNA. The final concentration of each primer in the PCR tube will be 0.2 μ M.

21. Very carefully, pipette 1 μ l of your isolated cheek cell DNA into your PCR tube.

You may use a P20 pipettor even though it is generally used only to 2 μ l.

22. Mix the contents of your PCR tube well by gently flicking the bottom of the tube. Then, vortex the tube gently (at a vortex speed of 3) until the bead has gone completely into solution. The contents of the tube should be clear.

23. Microfuge the tube briefly (5 seconds) to get all the contents of the tube to the bottom.

You will need to use adapters to microfuge these tiny tubes; your lab instructor and TA will have information about how to do this.

24. Follow the instructions of your lab instructor and TA for loading your sample into the PCR machine (called a "thermal cycler"); you can mark your tube, but you cannot rely completely on the mark staying on during the temperature cycles. Your instructor or TA will tell you how to record the location of your tube in the thermal cycler.

Here is the cycle we will use for your samples:

First, there is an initial step for 5 minutes at 95°C, to make sure all the DNA strands are disassociated to begin with.

This is followed by 35 cycles with the following pattern:

1 minute at 95°C, for "melting" the DNA

1 minute at 55°C, for reannealing the DNA

1 minute at 72°C, for building new DNA

There is then a 10 minute stage at 72°C, to complete whatever building has been started.

Finally, the tubes are chilled at 4°C until someone moves them to the freezer for longer term storage.

25. Your lab instructor or TA will return to lab after the PCR cycles are complete. They will store your PCR tubes in a box in the freezer.

HUMAN CDK3 SNP LAB II.

RESTRICTION ENZYME DIGEST AND GEL ELECTROPHORESIS

Project Goals

Last week in lab, you isolated your own DNA from cheek cells and set up a PCR reaction. The PCR amplified a region of DNA which contains a SNP; the SNP we are investigating is from an intron in the Cdk3 gene. This week, you will attempt to digest your PCR product with a restriction enzyme which recognizes one variant of this SNP but not the other variant. You will gain an understanding of using PCR to amplify a specific segment of DNA and using RFLP to detect different forms of a SNP.

Additional Reading

Review the background information presented on pages 1-2 of last week's handout. If you didn't have time to answer the questions throughout the background information, you will find that helpful before lab this week.

Experimental Procedures:

Work in groups of three today. Be prepared to start your restriction enzyme digest when you first come into lab. It will require a 90 minute incubation.

Restriction Enzyme Digest

1. Locate your PCR tube from last week, and set it out at your bench to thaw. Your lab group (3 students) will need to thaw one tube of 10X restriction buffer ("10X RB").
2. Each person at your lab bench will set up a restriction enzyme digest with their own PCR product; in addition, as a group, you will set up one digest of cheek cell DNA from a PCR tube your TA set up last week.

Your TA performed PCR using cheek cell DNA previously isolated from a heterozygous cheek cell donor; this sample will serve as a "known" lane on your gel.

3. Get a clean microfuge tube and label it with your name or initials, and some indication that you are digesting the DNA in the tube. Your lab group should label one tube for the sample your TA will share with you.
4. Pipette 8 μ l of your PCR product into your microfuge tube. Someone in your lab group should pipette 8 μ l of your TA's PCR sample into the proper labeled tube.

Be very careful not to pipette more than 8 μ l; you will need most of the remainder of your PCR product to run uncut on your gel.

5. Add 1 μ l of 10X Restriction Buffer to your tube. Mix well by pipetting up and down. Someone in your group should repeat with the sample from your TA.

You may use a P20 pipettor even though it is generally used only to 2 μ l.

6. Your TA will add 1 μ l of the restriction enzyme Hpa II to each of the microfuge tubes from your group.
7. Find a floater for your microfuge tubes, and put all four tubes from your group (one for each member of your group plus one tube with the TA's sample) in one floater. Incubate your tubes in the 37°C water bath for 90 minutes.

DO NOT THROW AWAY YOUR PCR TUBE: you will run uncut DNA on your gel along with the product of your restriction enzyme digest.

Prepare to Run a Gel

8. Making an agarose solution:
 - a. Each lab group should make a 40 ml solution of 2% agarose.
 - b. Calculate the amount of agarose you should weigh out to make your solution (you can do this before lab):
40 ml 2% agarose:

weigh out _____ g of agarose
dilute in _____ ml of TBE buffer

Remember, use the fact that 1 g is equal to 1 ml to do this calculation. Measuring volume to the nearest milliliter is fine when you are making agarose for a gel.

- c. Check your calculations with your lab instructor or your TA before proceeding.
- d. Using one of the balances in lab, carefully weigh out the appropriate amount of agarose, and mix it in the proper amount of TBE buffer (not water).

It will not go into solution until the next step.

- e. Microwave your agarose solution, checking the flask often to prevent boiling over. Use the “hot hands” to handle the flasks, since the glass will get very hot.

The agarose should be completely dissolved and the solution should be uniformly clear when you swirl it around.

- f. Take your flask back to your bench and let it cool until you can comfortably touch the glass flask.

You can do step 10a while you are waiting.

9. Adding GelStar DNA stain to your agarose:

- a. After your flask of agarose has cooled enough that you can touch it near the top comfortably, it is time to PUT ON GLOVES.

Any time you work with your agarose or gel for the remainder of the lab, you will need to be wearing gloves.

- b. Ask your TA to add 4 μ l of GelStar stain to your flask. Wearing gloves, swirl the flask gently to mix, and proceed to the next step.

10. Casting a gel:

- a. Wearing gloves, raise the sliding gates of your gel-casting tray and fasten them in place by gently tightening the plastic screws at either end. Place the 8-well plastic comb into the slots nearest the end of the gel casting tray.

Place the prepared tray on a level, undisturbed location on your bench so that when you pour the liquid agarose solution into the gel-casting tray it can solidify unperturbed. You will be using a comb which makes 8 wells this week, rather than the 6-well combs you have used previously.

- b. Wearing gloves, pour the agarose solution into the prepared casting tray, watching to make sure the agarose does not overflow.

You may not need to use all the agarose you have prepared

If you pour the agarose while it is too hot, you can crack or warp the casting tray, which may cause leaking.

- c. After pouring, check to make sure that there are no large bubbles in the molten agarose. If there are bubbles, use a clean pipette tip to move the bubbles to the side of the tray.
- d. Leave the gel alone to harden for several minutes while you go on to the next section.

11. Preparing your undigested samples:

- a. Label a microfuge tube with your initials and indicate that it contains undigested DNA.
- b. Pipette 10 μ l of your PCR product from your PCR tube into the microfuge tube.

If you do not have 10 μ l left, add as much of your PCR product as you have.

- c. Add 2 μ l of loading dye to the microfuge tube. Mix thoroughly by pipetting up and down. Don't forget to use a clean, new tip for each tube.

Do not throw the stock tube of loading dye away when you clean up after this lab.

12. Preparing the marker lane:

- a. Your lab group should acquire one tube of marker lane DNA from your lab instructor or TA.

This marker lane DNA is a 100 bp ladder rather than the 1000 bp ladder we have used on our other gels this term. It contains DNA fragments of the following sizes: 1517 bp, 1200 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, and 100 bp. Using what you know about the SNP region we amplified with PCR and the location of the Hpa II cutting site, you should be able to predict where you might see bands with respect to the marker lane bands.

- b. Add 2 μ l of loading dye to the marker lane DNA tube. Mix thoroughly by pipetting up and down.

13. Transferring your gel to the gel box:

- a. Once your gel has solidified, remove the comb carefully.

- b. Make sure the gates on your tray are down, and place the casting tray, still containing the gel, into the gel rig with the proper orientation: the comb end of the gel should be closest to the black (negative) electrode.

If there is already buffer in the rig, you can leave it there and re-use it (as long as the rig has been covered to prevent evaporation).

- c. Add enough TBE buffer to completely cover the gel.

Do not fill the gel rig completely (it will run too slowly); just make sure the gel is completely submerged.

Do not begin loading samples into your gel until all the samples (including those digested with Hpa II) are completely ready to be loaded.

14. Preparing your digested samples:
 - a. After your restriction enzyme digest is complete, microfuge your tubes briefly (15 seconds) to get any condensation from the lid into the bottom of the tube.
 - b. Add 2 µl of loading dye to each tube of digested DNA (one tube for each member of your lab group, plus the tube containing your TA's sample).

You should now (assuming you have 3 members in your group) have eight tubes which contain DNA and loading dye: one digested tube for each group member, one undigested tube for each group member, one digested tube of sample from your TA, and one tube of marker lane DNA.

Loading and Running the Gel

15. Load the contents of each tube into the gel. Be careful not to let any bubbles into the pipette tip when you are drawing the sample into the tip.

Check your tubes before loading: microfuge briefly (around 15 seconds) if necessary. In your notebook, make a record of which sample is loaded into which well.

16. When all of the samples have been loaded, re-check the orientation of the gel with respect to the electrical leads. Remember that DNA, having a

net negative charge, will migrate toward the positive (red) electrode.

17. Placing the lid on the box connects the leads to the gel box. Connect the other ends of the leads to the power supply. Check that the power supply is set at 100-105 volts, and then turn on the "juice". The voltage will be applied to the gel for 55 minutes.

Viewing the Gel

18. When the gel has run for 55 minutes, disconnect the leads from the power supply and remove the lid from the gel rig.
19. Carefully, wearing gloves, remove your casting tray (still containing the gel) from the gel rig and set it on your benchtop.
20. Remove the gel from the casting tray and transfer it to the plastic sandwich container at your bench. Do not allow the gel to flip over as you make the transfer. Carry the gel over to the sink, and rinse it gently with distilled tap water (black knobs at the sinks) to remove any unbound stain from the gel. When this destaining has been completed, you will view your gel on the transilluminator (UV light box). Your TA or lab instructor will help you with this.
21. Ascertain whether your enzymes cut your DNA. How can you tell? How many fragments do you see for each person in your lab group?

Recording Your Data

22. Determine what genotype you are: AA, AG, or GG. Be sure to check your interpretation of the gel with your lab instructor or TA. Once you are sure what your genotype is, write this on a 3x5 card (available in lab) and put the card in the envelope specified by your instructor or TA. Do not write your name on the card.

Class results will be compiled and presented to protect anonymity. You are not required to share your personal data with anyone else in lab (though you are free to do so if you wish).