# Screening recombinant clones (patch, PCR, digest)

NOTES: There are many strategies available for screening recombinant clones. The easiest strategy is when the recombinant has an obviously different phenotype to the vector plasmid alone – e.g. if you are cloning a resistance gene or a fluorescent protein. However, in most cases, we will need to screen the clones by PCR to find the recombinants that we are seeking.
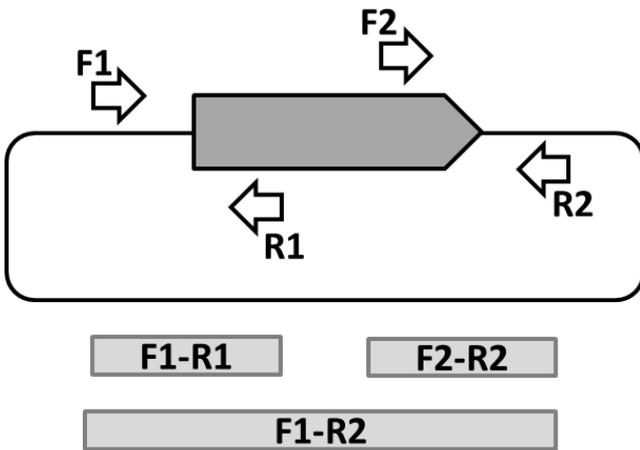
The diagram shows some PCRs that can be used to determine if a plasmid clone is:
   A. The desired recombinant
   B. Some other kind of recombinant
   C. The vector only

The PCR using primers F1-R1 is the best starting point for screening– this is the 'left junction' PCR. If you get a product of the expected size in this PCR, this is a good indication that you have the desired recombinants. The PCR using primers F2-R2 (right junction PCR) can then be done on clones that are positive in the F1-R1 PCR. If the clones are also positive in this PCR, this is further evidence that they are the right thing.

The PCR using primers F1-R2 is known as a 'spanning' PCR or 'insert spanning' PCR – this will amplify the whole insert region. This PCR can be used to determine if the clones have an insert of the expected size, but note that this is not an all or nothing PCR, unlike the junction PCRs. In the spanning PCR, the clones will give a small product if they have no insert, and a large product if they have an insert. This PCR is a bit ambiguous though, since if you have a mixed population of cells in your template DNA, you may see only the small product (this is amplified preferentially), even if you do have some positive clones in there too. The spanning PCR is most useful in preparation for sequencing the insert DNA region, rather than for initial clone screening.

The primers used for amplification of the original DNA insert (not shown in diagram) can also be used for screening PCRs. e.g. these primers used on their own can tell you if the insert DNA is present. (but they wont tell you which way around it has been cloned, unlike the junction PCRs). You can also do a junction PCR using one of the original primers, and a primer in the vector on the other side of the insert DNA, although this may give large products, and a less reliable PCR compared to using dedicated junction screening primers.

The positioning of the screening primers is important for later steps (sequencing). Make sure that the primers F1, R1, F2, R2 are at least 100 bp away from the ligation join – this is because the first ~50 bp of sequence read are usually 'junk'. Also ensure that these primers are not *too* far from the ligation join, otherwise you are amplifying (and later sequencing) stuff that you don't need to, and it makes the PCR take longer to thermocycle. Placing the primers about 150-200 bp from the ligation join is good, which gives an overall PCR product size of 300-400 bp.

INITIAL PATCHING AND PCR SCREENING:

1. Choose 20 <u>well-isolated</u> colonies from your transformation plates. Circle these and number them on the base of the plate.

2. Label three PCR strip tubes with numbers 1- 20, leaving three blanks (one in each strip) as negative controls. (so your first strip should be clones 1-7, the next one should be clones 8-14, and the last one should be clones 15-20)

3. Using an agar plate containing the appropriate antibiotic, divide the plate into patches, using a square grid (5 x 5). Label twenty of the squares with numbers 1 to 20. (see diagram)

2. Prepare a PCR master mix with the appropriate <u>left junction PCR</u> primers, enough for ~30 PCRs, aliquot this into 3 x 8 lots of 25 µl in the labelled PCR strip tubes. Taq polymerase is good for this job (it is very robust to 'mess' in the PCR) but other polymerases can also be used.

3. Using a white pipette tip, pick up some growth from the colony of your first clone of interest. Make sure you can see some cells on the tip, but you don't need a lot (approx 1 mm^3 = 1 µl). Dip the tip in and out of the mix in the first PCR tube five times. You don't need to dislodge the whole chunk of growth, enough cells will fall into the mix to give you a product, if the gene of interest is there. (this is called a 'colony PCR')
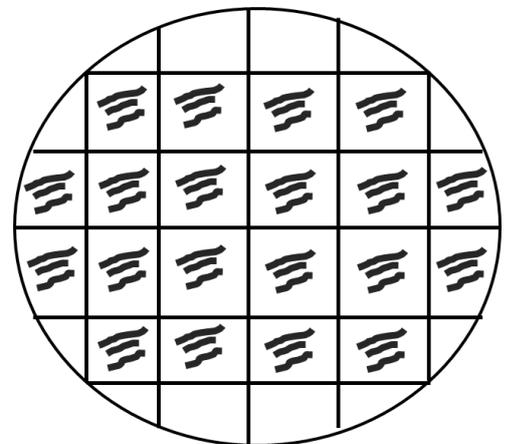
4. <u>With the same tip</u>, transfer the remaining growth onto the first patch on your agar plate, by scratching the tip across the surface of the agar a few times (e.g. make a set of three closely-spaced parallel lines – see diagram). Stay well clear of the borders of each square, we don't want the clones to touch each other when they grow up. Then discard the tip.

5. Repeat steps 3 and 4 with the remaining 19 clones, being very careful to keep track of which colony, which tube, and which patch you are up to (labelling <u>before</u> you start is important!)

6. Put the PCRs on the thermocycler and incubate the agar plate at 37°C overnight.

7. Run out the PCRs on an agarose gel, and note which clones give a positive result.

> If none of your clones are positive, you can try screening more clones (e.g. another 20), but if you still don't find your clone of interest after screening ~40 clones, something is wrong – check all your ligation and transformation controls, check all your primer sequences and thermocycling conditions. You may need to repeat the cloning process. It is also possible that the gene you are trying to clone is toxic to the host cells – you may need to try a different vector or different host or different incubation conditions.

SECONDARY PCR SCREENING

1. Examine the patch plates prepared above Choose up to seven of the clones that gave a positive PCR in the left junction screen (at least three is recommended).

2. Set up a PCR master mix using the <u>right junction</u> primer set. Remember to make enough master mix so that there is at least 10% more than you need, so you don't run out.

3. Screen the putative positive clones as described above, by colony PCR. Again, be careful to label everything before you start. Pay close attention to which clone patch is going into which PCR tube.

4. After thermocycling, run out the PCRs on agarose, and note which are positive for the right junction. Choose one of these for a plasmid prep; use this clone to inoculate 100 ml LB-antibiotic broth (see other protocol for details of plasmid prep).

5. <u>Keep the patch plate</u>! (wrap in parafilm, and store in cold room) – you may need to come back to this plate to plasmid prep a different clone if there are problems with the first one.

VALIDATION OF RECOMBINANT PLASMID BY RESTRICTION DIGESTION

NOTES: When making recombinant clones, we need to have several lines of evidence that the new plasmid has the correct structure. It is not sufficient to rely solely on the junction PCRs. For example, if the same insert has been cloned twice (head to tail) into the vector, of if there is a deletion in the middle of the insert DNA, the junction PCRs will look fine, but the overall structure will be wrong. Doing digests of the new plasmid provides another line of evidence for the correct structure, and also it allows us to quantitate the plasmid accurately, which is usually needed in any case for further work.

1. Using the plasmid prep from your chosen recombinant clone (above), set up a number of restriction digests which will be diagnostic of the correct structure. For example, you can choose an enzyme that cuts in the insert DNA but not in the vector DNA (this is ideal), and/or you can use an enzyme that cuts in the vector only (this is still OK, if the insert is quite large i.e. >500 bp). You want to be generating between 1 and 3 restriction fragments in total, and you want the recombinant clones have a clearly distinct digest pattern to 'vector only'. You want to be doing two or three different kinds of digests to give maximum confidence in the structure of the plasmid, not just one digest.

2. See other protocol "Protocol for quantitation and restriction digestion of plasmid (gel analysis)" for details of how to set up these plasmid digests.

3. If the chosen plasmid clone looks correct based on both junction PCRs and restriction digests, then you can proceed to sequencing (below).