The Principle

SW102 bacteria contain a defective phage expressing the lambda red genes which mediate homologous recombination between DNA stretches as short as 30bp. The genes are under temperature sensitive control – they are turned off when grown at 32°C or can be induced by shifting the bacteria to 42°C for 15 minutes. The recombination genes do not survive freeze-thawing well, so bacteria must be made competent on the day that you use them.

We use a selectable cassette encoding lacZ, kanamycin resistance & rpsL. In the first round of recombineering you amplify the cassette with 100bp primers – each primer has approx 20bp of homology to the kan/rpsL/lacZ cassette at the 3' end, and ~80bp arms of homology to the target insertion site in the BAC at the 5' end.

Take your SW102 bacteria containing the BAC you want to modify & grow overnight. The next day, grow the bugs to competency (3hrs), induce the lambda red genes (15mins) & wash the bugs to get rid of media. Electroporate your PCR product into the bugs, recover & select on media containing kanamycin, X-gal & IPTG. Wait for blue colonies to grow – these will have the cassette integrated into the BAC in the intended place according to your arms of homology.

In a second round of recombineering, you do the same again but instead of amplifying the rpsL/kan/lacZ cassette you amplify the sequences you want to insert into the BAC. There are 3 types of DNA construct you may want to use in this second round:

1. A PCR product – this may be a promoter, GFP ORF, whatever you want. Again, use 100bp primers with ~20bp homology to the sequence to be inserted at the 3' end & ~80bp arms of homology to the target insertion site on the BAC.

2. An oligo - if you're only adding a few bases, or just deleting sequences then no PCR is required, just order an oligo with at least 40bp homology to the left of the deleted/inserted bases, any changed bases & at least 40bp homology to the right of the deleted/inserted bases.

3. 2 Oligos - if you want to add in slightly more sequence than you can incorporate in a 100bp oligo, you can use 2 oligo's – a 'forward' and a 'reverse' oligo. Design the oligo's so that the forward one has homology to the region of the vector that is upstream of the inserted bases at the 5' end, then the inserted bases. The reverse oligo has homology to the region of the vector downstream of the inserted bases at its 5' end, then the inserted bases. Thus the 2 primers overlap & are complementary to each other at their 3' ends. As long as the 3' complementary overlap between the 2 oligos is ~25 bases, you can just electroporate the 2 oligos in, and the bacteria will fill in the missing strands.

Again make the bugs (this time containing the BAC with the kan/lac/rpsL cassette inserted) competent and this time electroporate in your cassette/oligo. Select on media containing streptomycin, X-gal & IPTG. rpsL makes E.coli sensitive to streptomycin & so kills bugs where the rpsL cassette hasn't been replaced by your sequences. Sometimes rpsL mutates, so the bugs still grow even though they are wrong. LacZ lets you screen these out – in the correct colonies lacZ is also replaced, so colonies are white, in the colonies where sacB has mutated, LacZ is still present so the colonies are blue.

Pick a white colony & sequence it, and you're done!

Obviously it's a bit more involved – full protocols below.

Day 1 - PCR

The biggest primer you can order is 100bp – we order desalted primers from invitrogen. The size of the region of homology affects the efficiency of the recombination, so add as much of the homologous sequences as you can to the ends of your primers. See the kan/rpsL/LacZ sequence for the cassette specific bits you will need to add to your primers (marked Primer-L and Primer-R, at either end of the sequence).

> **Note** Most companies recommend PAGE purifying primers of this length. We have no problems with just desalted primers.

1. Amplify the rpsL cassette using Roche Expand Hi-Fi. Use buffer 2 (contains Mg) and 0.5µl enzyme per 50µl reaction. Use the following program:

   95°C 2 min
   35 cycles of 95°C 30sec, 55°C 30 sec, 72°C 2min
   72°C 15 min

   > **Note**. Proof readers don't work so well with these longer primers. We've had some success with NEB's Phusion, when adding 3% DMSO. However it still only works about 33% of the time.

2. Add 1µl DpnI & incubate for 30-60 mins @37°C. This destroys methylated DNA (i.e. the PCR template) but leaves the amplified product alone.

3. Run your entire PCR on a gel, and gel purify it. We use Amersham GFX columns, but any kit should work. Elute in ~30µl water, you can use as low as 10ul to concentrate it if the PCR didn't work well.

   The columns generally have a maximum volume of ~600µl. You'll probably find that you have a greater volume than that after melting your agarose slices. In this case, apply 600µl, spin and discard the flow-through and then apply more of the same sample to the same column & spin again. Repeat as needed before continuing with the protocol.

   > **Note.** The amount of DNA doesn't seem to be too limiting – I've done parallel experiments using approx 400ng or approx 20ng PCR product, and got comparable numbers of positive colonies at the end.

Day 2 - First round of recombineering (positive selection)

See end for media & antibiotic recipes.

1. The previous day, inoculate some SW102's containing the desired target plasmid into 5 ml LB + chloramphenicol (12.5μg/ml). Incubate overnight at 32°C.

2. Inoculate 0.5ml of the overnight culture into 25ml LB in a 50ml falcon (I generally leave out antibiotics to encourage the bugs to grow faster). Incubate at 32°C in a shaking incubator to an OD_{600} of approx. 0.6 (I've overrun to as high as 0.75 & it's still worked). This usually takes 3-3.5 hours.

   During this incubation, put at least 75ml sterile ddH_2O into the fridge, turn on a waterbath to 42°C and turn a centrifuge that can take 50ml falcons down to 0°C.

3. When the culture has reached its OD, induce the lambda red proteins by incubating in the 42°C waterbath for 15 minutes. As long as the lid is screwed down, just float the falcon on the surface. Invert the falcons a couple of times over the 15 minutes to ensure good heat mixing.

4. Centrifuge for 5 minutes @4000rpm, 0°C. It's important to keep the bacteria as close to 0°C as possible in order to get good competent cells.

5. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold ddH_2O by gently swirling the tubes. When resuspended, add another 25 ml ice-cold ddH_2O and spin the samples again.

6. Repeat the washing step

7. Pour off all supernatant. Be careful – the cell pellet is very loose by now. Resuspend the pellet in the remaining water (~400μl) by gently shaking it.

8. Transfer the competent SW102's into 0.5ml eppendorfs as 25μl aliquots. Add ~3μl purified PCR product to a 25μl aliquot of induced bacteria.

9. Transfer aliquots +DNA to pre-cooled cuvettes and stand on ice for 5 minutes. For 0.2cm cuvettes, electroporate using the EC3 program, for 0.1cm, use the EC1 program.

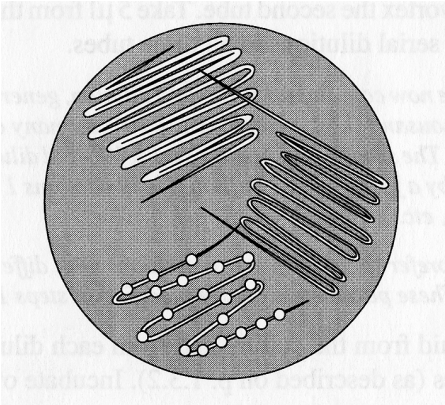
10. Recovered bacteria in 1ml LB for 1hr at 32°C in a shaking incubator.

11. Spread 200μl onto LB + kan + chlor + IPTG + X-gal.

12. Wait for blue colonies to form – this takes ~24 hours due to there being only a single copy of lacZ per cell and the fact that the bugs grow slower at 32C.

Day 3 - Checking the colonies

In the second recombineering you will be selecting against the sacB, therefore you need to check that rpsl is functional – occassionally PCR errors will prevent it working. So inoculate a colony into some water and plate equal amounts onto a LB+kanamycin plate & a LB+streptomycin plate. Spread for single colonies like this:



Also inoculate into 5ml LB. Do this for 4 different blue colonies. Grow LB & plates overnight @32C.

Day 4 – miniprep, digest & sequence

If the rpsL is working, there should be a clear difference in the growth of colonies on the 2 plates. For all colonies where the rpsL is working, miniprep:

Miniprep

You get VERY little DNA out of a miniprep. You can miniprep using standard miniprep kits, but you won't get enough DNA out to sequence, or see very well on a gel. Hence this method.

1. Pellet ~4.5 mls overnight culture in a 15ml falcon (4000rpm, 5 mins). We use buffers from the qiagen spin miniprep kits to do an alkaline lysis - resuspend in 250 μl buffer P1 and transfer to a 1.5ml eppendorf tube.

2. Add 250 μl P2, mix and incubate for 5 min at room temperature.

3. Add 250 μl N3 buffer, mix. Because of the amount of precipitate you may have to invert several time.

4. Centrifuge at full speed for 10 min. and transfer supernatant to a new tube.

5. Precipitate DNA by adding 750 μl isopropanol, mixing and centrifuging for 10 min. at full speed @4°C. This should give you a visible pellet.

6. Remove and discard supernatant, then add 500μl 70% ethanol. Centrifuge for 10 min at full speed.

7. Remove and discard supernatant, airdry & redissolve in 30μl tris/water.

All the colonies you get from positive selection should be correct, but I like to do a restriction digest to check:

Restriction Digest

1. Combine 8μl DNA, 1μl buffer, 1μl BamH1 and incubate for an hour at 37°C.

2. Add loading buffer and load the entire 10μl on an agarose gel.

HindIII, BamH1 & EcoRV all give recognisable banding patterns for the Merlin BAC.

As long as the gross restriction pattern looks correct, continue on to the second round.

Day 7 – second round of recombineering (Negative Selection)

Do the recombineering as before:

1. The previous day, inoculate some SW102's containing the desired target plasmid into 5 ml LB + chloramphenicol (12.5μg/ml)+ Kan (15ug/ml). Incubate overnight at 32°C.

2. Inoculate 0.5ml of the overnight culture into 25ml LB in a 50ml falcon (I generally leave out antibiotics at this stage). Incubate at 32°C in a shaking incubator to an OD_{600} of approx. 0.6 (I've overrun to as high as 0.75 & it's still worked). This usually takes 3-3.5 hours.

   During this incubation, put at least 75ml sterile ddH_2O into the fridge, turn on a waterbath to 42°C and turn a centrifuge that can take 50ml falcons down to 0°C.

3. When the culture has reached its OD, induce the lambda red proteins by incubating in the 42°C waterbath for 15 minutes. As long as the lid is screwed down, I just float the falcon on the surface. Invert the falcons a couple of times over the 15 minutes to ensure good heat mixing.

4. Centrifuge for 5 minutes @4000rpm, 0°C. It's important to keep the bacteria as close to 0°C as possible in order to get good competent cells.

5. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold ddH_2O by gently swirling the tubes. When resuspended, add another 25 ml ice-cold ddH_2O and spin the samples again.

6. Repeat the washing step

7. Pour off all supernatant. Be careful – the cell pellet is very loose by now. Resuspend the pellet in the remaining water (~400μl) by gently shaking it.

8. Transfer the competent SW102's into 0.5ml eppendorfs as 25μl aliquots. Add ~3μl purified PCR product or 1μl oligos to a 25μl aliquot of induced bacteria.

9. Transfer aliquots +DNA to pre-cooled cuvettes and stand on ice for 5 minutes. For 0.2cm cuvettes, electroporate using EC3 program, for 0.1cm use EC1.

10. You need to remove the bugs & incubate them in 5ml LB. A pipette won't fit down into the cuvette, so put 5ml LB into a universal, then use a 1ml pipette to take up 1ml LB, add it to the cuvette to 'wash' the bacteria out, then suck up the whole 1ml+E.coli and transfer back into the universal.

11. Recover bacteria in 5ml LB for 4hrs at 32°C in a shaking incubator.

Note. This is different from round 1 – with negative selection you need to allow time for the rpsL proteins to disappear from the cell

12. Spread 150µl on LB + streptomycin + chlor + X-gal + IPTG (see later for recipe). Wait 48 hours for white, sucrose resistant colonies to grow. The colonies need to be fairly well spaced for the blue colour to develop nicely.

Day 9 – set up miniprep

Pick 4 white colonies per insert & grow in 5ml LB overnight.

Day 10 – miniprep, digest, sequence

Miniprep as before, digest again & run a gel. You sometimes get colonies where funny recombinations have occurred. These are normally very obvious – e.g. you were expecting 15 bands but only get 2. If the digest pattern looks right, sequence your insert. Youn won't be able to sequence your BAC directly, the amount of DNA is too low. Instead, design some primers that bind outside the 'arms of homology' from your recmbineering primers, PCR up that region, purify it from solution & send the PCR product for sequencing (using the PCR primers to sequence it).

Day 12-ish - maxiprep

You really need to maximise bacterial growth – you should be able to get ~40μg total DNA from 500ml culture.

1. It's important to maximise bacterial growth. Inoculate a single colony into 5ml LB + chlor in the morning, allow it to grow during the day (32°C)

2. Dilute all 5mls in 500ml LB then grow overnight at 32°C in a large flask (volume >4× the volume of media).

3. Prepare DNA using the BacMAX 100 kit (Machery Nagel). Follow the large construct instructions (i.e. resuspend in 24ml of buffer S1, lyse in 24ml buffer S2 etc).

   > **Note.** The instructions offer 2 ways of clearing the bacterial lysate. Filtration is kinder to the large BAC DNA, & quicker than centrifugation, however it helps if you give the lysate a quick spin (15 mins @6,000 rpm) before putting it onto the filter.

4. When eluting DNA from the columns at the end, pre-warm the elution buffer to 50°C.

5. At the end, resuspend DNA in 100μl 10mM Tris pH8.5. Concentration should be 200-400ng/μl.

Other info

Media

We use low salt LB:

10g/L tryptone
5g/L salt
5g/L yeast extract

Antibiotics

Use chloramphenicol at 12.5µg/ml
Use kanamycin at 15µg/ml
Use streptomycin at 1200ug/ml

Positive Selective plates

For positive selection, LB + chlor + kan + X-gal + IPTG

To 500ml autoclaved (& cooled to ~50°C) LB Agar add:

1ml X-gal (40mg/ml stock conc)
1ml IPTG (100 mM stock conc)
500µl chloramphenicol (12.5mg/ml stock conc)
500µl kanamycin (15mg/ml stock conc).

Negative Selective Plates

For negative selection, to autoclaved & cooled LB agar, add:

1ml X-gal (40mg/ml stock conc)
1ml IPTG (100 mM stock conc)
500µl chloramphenicol (12.5mg/ml stock conc)
1500ul streptomycin (400mg/ml stock)

Things that effect cloning efficiency

Competency of cells

More competent cells = more correct colonies

Size of insert

Larger inserts clone with lower efficiency – there's nothing you can do about this, and because of the blue/white screening it's not a real problem, but you will get fewer white colonies from a larger insert.

Other selectable cassettes

There are other selectable cassettes you can use – in particular the lab who supply the SW102 bacteria use a galK cassette. It offers the advantage that you select for & against the same cassette, and it doesn't mutate as much as rpsl. However it requires minimal media to be made & is generally a bit more hassle, so we stick with the rpsL cassette method.

We also have a cassette that uses amp instead of kan, and sacB instead of rpsL. Counterselection here is on 5% sucrose, using media lacking salt. However the cassette is larger and so inserts less efficiently than the rpsL cassette, so offers little advantage.