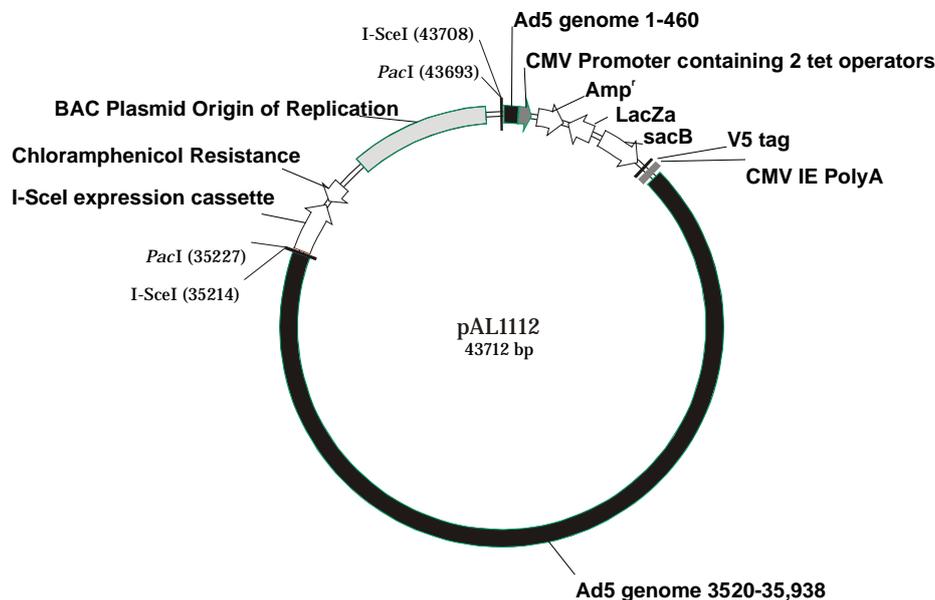


## The Principle

The AdZ-5 vectors contain the Ad5 vector genome, deleted for E1 & E3 regions (rendering the vector non-replicative) in a single copy vector. It contains the HCMV major immediate early promoter & PolyA signal sequence, with a cassette in the MCS encoding ampicillin resistance, lacZ $\alpha$  & SacB. SacB encodes for a gene giving sensitivity to sucrose, lacZ $\alpha$  provides blue/white screening.

The AdZ CMV promoter is repressible by tet repressors, thus if the vector is grown in 293TREx cells expression from the CMV promoter is prevented during growth. This allows the cloning of toxic genes, or genes which would otherwise interfere with the Ad replication. Expression from the CMV promoter in cell lines not expressing the tet repressor (ie most other cell lines) is constitutively on.



SW102 bacteria contain a defective phage expressing the lambda red genes which mediate homologous recombination between DNA stretches as short as 50bp. 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. Unfortunately the recombination genes do not survive freeze-thawing well, so bacteria must be made competent on the day that you use them.

## Protocol outline

PCR your gene using primers that contain 50bp+ regions of homology to the CMV promoter & polyA. SW102 bacteria are made competent, the recombination genes induced and then the PCR product electroporated into the bugs. If recombination occurs, the amp-lacZ-SacB cassette will be replaced by your gene. Bacteria are selected on plates containing sucrose so that any bacteria where recombination hasn't occurred (and therefore still contain sacB) will die. Background bacteria where sacB has mutated (and therefore non-functional) will also appear. lacZ $\alpha$  allows you to screen these out – blue colonies contain mutated sacB, white colonies will contain the correct insert.

## AdZ-5 vectors

These vectors are based on wildtype adenovirus type 5 virus kindly provided by Vivien Mautner from Birmingham University.

All vectors are Ad5  $\Delta$ E1 (461-3519bp),  $\Delta$ E3 (28131-30,800bp) (deletion numbering based on the prototype Ad-5 sequence (AC000008)).

| <i>Vector</i> | <i>Tet-operators in promoter?</i> | <i>Self Excising?</i> | <i>Tag</i>                                       |
|---------------|-----------------------------------|-----------------------|--|
| pAdZ5-CV5     | Yes                               | Yes                   | C terminal V5                                    |
| pAdZ5-NV5     | Yes                               | Yes                   | N terminal V5                                    |
| pAdZ5-NGFP    | Yes                               | Yes                   | N terminal eGFP                                  |
| pAdZ5-CGFP    | Yes                               | Yes                   | C terminal eGFP                                  |
| pAdZ5-CCherry | Yes                               | Yes                   | C terminal mCherry                               |
| pAdZ5-miR155  | Yes                               | Yes                   | miR-155 arms of homology<br>(for cloning shRNAs) |
| pAdZ5-CStrep2 | Yes                               | Yes                   | C terminal StrepII tag                           |
| pAdZ5-CV5-NT  | No                                | Yes                   | C terminal V5                                    |
| pAdZ5-CGFP-NT | No                                | Yes                   | C terminal eGFP                                  |

## PCR

We order 100bp desalted primers from Invitrogen. The size of the region of homology affects the efficiency of the recombination, so add as much of the following homologous sequences as you can to the ends of your primers.

**Note** Most companies recommend PAGE purifying primers of this length. With desalted primers of this length we have occasionally seen primers with bases missing. If you want to be guaranteed of the sequence first time, you can get the primer PAGE purified. However this costs quite a lot more and in our experience primer errors are rare.

### If cloning your PCR product with no tag, use the following primers, and any of the vectors:

To your forward primer (this does not include a Kozak-optimized sequence, you may want to add your own):

5' AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGAC  
ACCGGGACCGATCCAGCCTGGATCC-**YOUR-PRIMER-HERE**-3'

To your downstream primer:

5' GGCCTGACACGTTTATTGAGTAGGATTACAGAGTATAACATAGAGTATAATATAG  
AGTATACAATAGTGACGTGGGATCC-**YOUR-PRIMER-HERE**-3'

### Cloning with a C terminal V5 tag

pAdZ5-CV5 and pAdZ5-CV5-NT contain a C-terminal V5 tag. If you want to clone a gene with this tag, use the same arm of homology as for untagged genes for the forward primer, and the following arm of homology for the reverse primer (tag is in bold, linker in italics):

5' - TATAGAGTATACAATAGTGACGTGGGATCC**CTACGTAGAATCAAGACCTAGGAGCGGGTTA**  
\*\*\*ThrSerAspLeuGlyLeuLeuProAsn

**GGGATTGGCTTACCAGCGCT**-**YOUR-PRIMER-HERE**-3'  
ProIleProLysGlyAlaSer

### Cloning with a N terminal V5 tag

pAdZ5-NV5 contains a N-terminal V5 tag. If you want to clone a gene with this tag, use the following arm of homology for the forward primer (tag is in bold, linker in italics). For the reverse primer use the same arm of homology as for untagged genes.

5' - CGGGACCGATCCAGCCTGGATCCCCACCAT**GGTAAGCCAATCCCTAACCCGCTCCTA**  
GlyLysProIleProAsnProLeuLeu

**GGTCTTGATTCTACGGGCGCC**-**YOUR-PRIMER-HERE**-3'  
GlyLeuAspSerThrGlyAla

### Cloning with a C terminal GFP tag

pAdZ5-CGFP and pAdZ5-CGFP-NT contain the entire eGFP ORF from pEGFP-N1 (Clontech) after the amp-lacZ-sacB cassette, so if you want a C terminal GFP tag use the following arms of homology:

For upstream primer, use the same arms of homology as for the untagged protein. For the downstream primer remove the stop codon from your gene & use the following. The beginning of the GFP ORF is in bold, there is a 9 amino acid linker that you can put in between your gene & the GFP (in italics) if you want to.

5' - **TCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATCGCGGA**  
*ValLeuIleProValValGlyThrPheLeuGluGluGlyLysSerValMetAlaSer*

*CCCTGCTGATCCAGCGCTACC* - YOUR - PRIMER - HERE - 3'  
*GlyAlaSerGlyAlaSerGly*

### Cloning with a N terminal GFP tag

pAdZ5-NGFP contains the entire GFP ORF from pEGFP-N1 (Clontech) before the amp-lacZ-SacB cassette. If you want an N terminal GFP tag, use the following arms of homology:

For downstream primer, use the same as for untagged inserts. For upstream primer use the following arm of homology. The end of the GFP ORF is in bold, there is then a 9 amino acid linker (in italics) that you can use to separate the GFP from your gene if you want to.

5' - **CTGGAGTTCGTGACCGCCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGTAGC**  
*LeuGluPheValThrAlaAlaGlyIleThrLeuGlyMetAspGluLeuTyrLysGlySer*

*GCTGGATCAGCAGGGTCCGCG* - YOUR - PRIMER - HERE - 3'  
*AlaGlySerAlaGlySerAla*

### Cloning with a C terminal mCherry tag

pAdZ5-CCherry contains mCherry after the sacB cassette – use the same forward primer as for untagged constructs, and the following arms of homology for the downstream primer:

5'CGCATGAACTCCTTGATGATGGCCATGTTATCCTCCTCGCCCTTGCTCAC  
CATCGCGGACCCTGCTGATCCAGCGCTACC - Your primer here- 3'

## Cloning with a C terminal streptag-2

pAdZ5-CStrep2 contains a C-terminal streptag after the amp-lacZ-sacB cassette, so if you are putting a C terminal streptag onto your protein, remove the stop codon from your gene & use the following arms of homology on the downstream primer (streptag is bolded, link is in italics) and recombine into pAL1240. For your forward primer, use the same regions of homology as for an untagged construct.

5' -TATAACATAGAGTATAATATAGAGTATAACAATAGTGACGTGGGATCC**TTATTT**  
\*\*\*Lys  
**TTCGAACTGCGGGTGGCTCCAAGCGCT-YOUR-PRIMER-HERE** -3'  
GluPheGlnProHisSerTrpAlaSer

## Cloning shRNA's

pAdZ-miR155 has miR-155 flanking arms of homology on either side of the SacB, Amp, LacZ selectable marker to allow shRNA insertion with oligonucleotides. The vector is based on a paper from David Turner's lab(1).

To clone an shRNA into pAdZ-miR155 design two oligos, a 'forward' and 'reverse' oligo that overlap in the centre by 25bp. Each oligo will have 40bp homology to the vector, followed by either sense or antisense sequence to your target, then a loop sequence. They will overlap through the loop sequence. The design is most easily described by an example. The 21bp sequence that we want to target in the cDNA is cgacagtatgatgaacacagt. The forward oligo contains 40bp of homology to pAdZ-miR155, 21bp antisense to the target sequence (green), a 19bp loop (red) and 3bp of sense sequence (blue).

The reverse oligo has 3bp of sense sequence, a 19bp loop (red), 19bp of sense sequence (blue) (with two bp missing from antisense sequence to form internal loop) and 40 bp of vector homology. This gives the oligo's 25bp overlap (the loop, plus 3bp either side) (note that the lower primer is written 3'-5' for clarity). The homology arms and loop sequence will be common to all shRNAs. We use the algorithm on the invitrogen website to design the appropriate oligos. <https://rnaidesigner.invitrogen.com/rnaexpress/setOption.do?designOption=mirna&pid=2084549680335976048>

Forward oligo:

5'-  
CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG**ACTGTGTT****CAT**  
**CATACTGTCGGTTTGGCCACTGACTGACCGA**

Reverse oligo:

3'-  
**AGC****CAA****AACCGGTGACTGACTGGCTGTCATTACTTGTGTCAGTCCTGTGTT**  
**CCGGACAATGATCGTGAGTGTACCTTGTT**

Writing the lower oligo in standard 5'-3' orientation would therefore give:

5'-

TTTGTTCCATGTGAGTGCTAGTAACAGGCCTTGTGTCCTGACTGTGTTTCAT  
TACTGTTCGGTCAAGTCAGTGGCCAAAACCGA

To clone the shRNA into the vector simply mix together 100ng (I normally dilute 100pmol/ul stock 1/30 and use 1.3 ul) of each oligo and transform into induced SW102 containing pAdZ5-miR155 and follow standard recombineering protocol. The insertion should be very efficient.

1. **Chung, K.H., C.C. Hart, S. Al-Bassam, A. Avery, J. Taylor, P.D. Patel, A.B. Vojtek, and D.L. Turner.** 2006. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res* 34:e53.

## PCR reaction

1. Do your PCR as normal using Roche expand Hi-Fi. This is a proofreading mix that is particularly robust when using the long primers required for recombineering. Use buffer 2 (contains Mg) and 0.5 $\mu$ l enzyme per 50 $\mu$ l reaction, and cycle for 30-35 cycles.

**Note.** Various proof-reading taqs have been tested for amplifying larger inserts (3kb plus). Phusion (NEB) has been used successfully when 3% DMSO was added. However for best results with Phusion consider doing a nested PCR with slightly shorter primers, which when added together give the longer arms of homology.

2. Run your entire PCR on a gel, and gel purify it. GFX columns (GE Healthcare) work well, and allow you to elute in small volumes if required. Elute in 30 $\mu$ l elution buffer, or 10 $\mu$ l if the PCR didn't work well.

The GFX columns have a maximum volume of 600 $\mu$ l. If the final volume of melted agarose is higher than this, apply 600 $\mu$ l, 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.

### Notes:

If you're amplifying from plasmid template then you can destroy the plasmid after the PCR reaction by incubating the pcr mix with DpnI for an hour. It cuts up methylated DNA (i.e. plasmid) but not unmethylated (PCR product). It works in the PCR buffer.

The amount of DNA is not limiting – parallel experiments using 400ng or 20ng PCR product gave equal numbers of positive colonies at the end.

## Making competent SW102's (see appendix 1 for media recipes)

1. Inoculate SW102's containing the desired plasmid into 5 ml LB + amp (50 $\mu$ g/ml) + chloramphenicol (12.5 $\mu$ g/ml). Incubate overnight at 32°C.
2. Inoculate 0.5ml of the overnight culture into 25ml LB + amp (50 $\mu$ g/ml). This will be enough for at least 8 inserts. Scale volumes up if necessary. Incubate at 32°C in a shaking incubator to an OD<sub>600</sub> of 0.6 (approx 3 hours).

During this incubation, put at least 150ml sterile ddH<sub>2</sub>O into the fridge, turn on a waterbath to 42°C and turn a centrifuge that can take 50ml falcons down to 0°C.

3. Pour the culture into 50ml falcons (without skirts). Induce the lambda red proteins by incubating in a 42°C waterbath for 15 minutes. Invert the falcon twice over the 15 minutes to ensure good heat mixing.

**Note** If desired, grow 50ml of culture, then split the culture in half and only induce one of the samples – leave the other at 32°C. This can serve as a negative control for recombination. In practice there is little need for this however.

4. Transfer the falcon into ice and cool for 15-20 minutes. If possible put the ice bucket on a gentle shaker to increase the rate of cooling.
5. Centrifuge for 5 minutes @4000rpm, 0°C.

**Note** It's important to keep the bacteria as close to 0°C as possible throughout the protocol in order to get good competent cells.

6. Pour off supernatant and resuspend the pellet in 1 ml ice-cold ddH<sub>2</sub>O by gently swirling the tube. When resuspended, add 25 ml ice-cold ddH<sub>2</sub>O and centrifuge the sample again.
7. Repeat the washing step
8. Pour all supernatant off. Be careful – the cell pellet is very loose by now. Resuspend the pellet in the remaining water (~400 $\mu$ l) by gently shaking it.
9. Transfer the competent SW102's into 0.5ml eppendorfs as 25 $\mu$ l aliquots. Add 3 $\mu$ l purified PCR product to a 25 $\mu$ l aliquot of induced bacteria.

**Note** As long as there is at least 10ng DNA, the concentration of DNA added is not important

10. Transfer aliquots +DNA to pre-cooled cuvettes and stand on ice for 5 minutes. We use 0.2cm cuvettes & electroporate at 2.50 kV, other people use 0.1cm cuvettes & electroporate at 1.8kV.

11. After electroporation recover bacteria in 5ml LB in a universal for 4 hours in a shaking incubator at 32°C. This extended recovery is needed to allow the loss of sacB DNA & proteins from the cells where recombination has occurred.

**Note.** If you find yourself running out of time, recover for as long as you can (at least an hour), then fridge the culture and finish the incubation off the next day.

12. Plate 50µl bacteria onto LB + 5% sucrose + chloramphenicol (see appendix for recipe; note NO ampicillin, correct bacteria are now ampicillin sensitive!) and grow for 30-48 hours at 32°C.

**Note** It is important to get well spaced colonies. The bacteria grow more slowly at 32°C than at 37 (hence leaving for a minimum of 30 hrs, not 24), and only have a single copy of lacZ per cell so it takes longer for the blue colour to develop. If they are too closely spaced, they don't grow well enough for the blue colour to be visible. Either plate out 50µl and examine after 24 hours, then replate a different volume if necessary or plate out 2 different dilutions to start with.

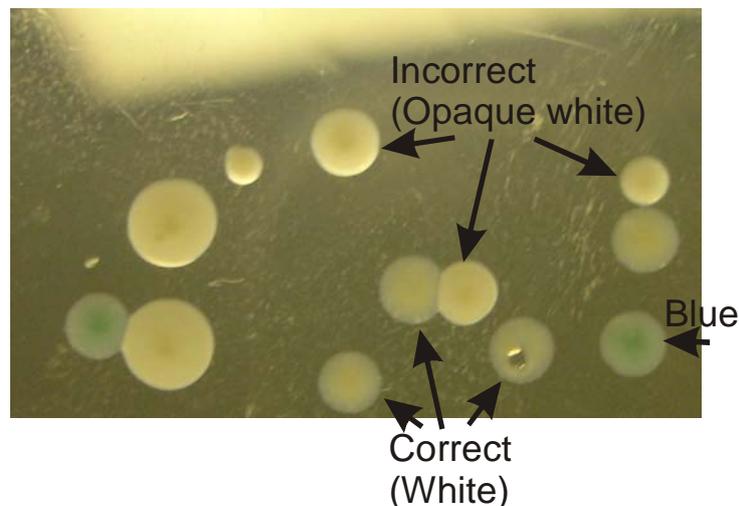
13. There should be plenty of white colonies among the blue ones.

**Note** Occasionally colonies are present that appear to be white but which still contain the amp/sacB/lacZ cassette. These false positives are easily avoided. Hold the plate up at an angle to a fluorescent light (not directly in front of the light, or you won't be able to see the difference). The false positives look much more opaque, and consequently whiter, under these conditions than the real positives. Once you've seen the difference it's quite obvious & you should have no problems picking the real white ones. If you do an uninduced sample of bacteria you can compare the uninduced plate to the induced using this method & you should easily be able to see that there is one sort of colony which only appears on the induced plate.

As an alternative leave the cultures in the fridge for 24-48 hours after the extended recovery. When you plate them out, the false positives will be gone, or much reduced.

**Note 2** PCR screens do not work very well – the low plasmid copy number means a lot of cycles are required to get product, which in turn can lead to false positives.

Here's what the 3 different sorts of colony look like:



## Minipreps

The AdZ-5 vectors are in a single copy vector – you only get ~100ng total DNA from a miniprep. The standard qiagen column miniprep kit results in too little DNA, hence this modified method.

1. Pick white colonies into 5ml LB+chloramphenicol & grow 16-20hrs @32°C. 4 different colonies for each insert should be more than enough.
2. Pellet 4.5 mls.
3. Using the qiagen spin miniprep kit, resuspend in 250 µl buffer P1 and transfer to a 1.5ml eppendorf tube.
4. Add 250 µl P2, mix and incubate for 5 min at room temperature.
5. Add 250 µl N3 buffer, mix. Because of the amount of precipitate you may have to invert several time – the lyseblue dye from the qiagen kit helps here.
6. Centrifuge at full speed for 10 min. and transfer supernatant to a new tube.
7. Precipitate DNA by adding 750 µl isopropanol, mixing and centrifuging for 10 min. at full speed @4°C. There should be a visible pellet.
8. Remove and discard supernatant, then add 500µl 70% ethanol. Centrifuge for 10 min at full speed.
9. Remove and discard supernatant, then airdry the pellet until the last of the ethanol has evaporated. Redissolve DNA in 30µl 10mM Tris pH8.5 (or buffer EB from the Qiagen kit).

## 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.

The AdZ-5 vectors give bands of 18, 11, 7.7, 2.5, 1.7, 0.8 and 0.6 Kbp. The 2.5 & 1.7 kbp bands are from the Sac/LacZ/Amp cassette, these should have disappeared & be replaced with a band corresponding to your insert. Often your insert size is small, and the amount of DNA quite low, so you won't be able to see your insert band. In this case, as long as only the 2.5 & 1.7 kbp bands have gone, the construct should be correct & can be sequenced.

## Sequencing

We use Big Dye 3.1 on an ABI sequencer.

1. Combine 5µl DNA, 1µl primer (3.2pmol/µl), 4µl BigDye mix.

**Note** You need to sequence your insert for PCR errors, but also the sequence corresponding to the arms of homology. This is in case your primer had any errors/deletions.

The following primers work well for sequencing into the insert, including the regions of homology:

From CMV Promoter: 5'- AATGTCGTAACAACTCCG -3'

From CMV PolyA: 5'-ACCTGATGGTGATAAGAAG -3'

2. Use the following program: 95°C for 5 minutes, then 100 cycles of 95°C for 30sec, 55°C for 10 sec, 60°C for 4 min.

**Note** Cycling for 100 cycles instead of the standard 25 is important – it gives much better results with low template concentrations.

3. Clean up the sequencing reaction using Performa DTR columns, according to manufacturers instructions.

**Note** You can just precipitate the DNA, but the low template concentration means there are a lot of dye terminator blobs at the beginning of the sequence. The DTR columns significantly reduce this.

## Maxiprep

250ml culture should give ~30µg total DNA.

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 250ml 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 standard maxiprep instructions (i.e. resuspend in 12ml of buffer S1, lyse in 12ml buffer S2 etc), with 2 modification from the 'low copy DNA protocol':

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.

Warm the elution buffer to 50°C before eluting DNA.

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

**Note** Culture volumes can be reduced if required. You only need enough DNA to do a single transfection (1-4µg depending on the transfection method).

## Making virus

Unlike other Ad vectors, the genome does not need to be digested before transfecting. Just transfect circular maxiprep DNA.

Any transfection method can be used. We transfect using Polyfect (Qiagen) as follows:

1. Seed  $2 \times 10^6$  293TREx cells (these can be bought from Invitrogen) into a T25 the day before transfection.

Note. If you aren't using the tet-repression system, you can use any cells such as 911, 293 or PERC6 to grow virus.

2. Dilute 4 $\mu$ g vector DNA in 100 $\mu$ l DMEM (no FCS or antibiotics)
3. Add 40 $\mu$ l polyfect and incubate 10 mins at room temperature. During the incubation, change media on the cells & add 3mls DMEM (containing FCS/antibiotics).
4. Add 1ml DMEM (with FCS/antibiotics) to DNA complex & transfer to cells.
5. After 24 hrs, change media. Keep changing it whenever media goes yellow. You should see plaques after 7-10 days.

## ***Appendix 1 - Selective plates & media***

Sucrose plates:

1. When making plates containing sucrose + chloramphenicol do not use salt; the lack of salt makes the bacteria a lot more sensitive to the sucrose:

10g/L tryptone  
5g/L yeast extract  
50g/L sucrose (i.e. 5%)  
15g/L agar

2. Dissolve some of the sucrose by warming to 37°C for 30 mins before autoclaving or it caramelises.
3. Autoclave, allow it to cool to hand-hot & add:

Chloramphenicol (1:1000 of 12.5mg/ml stock)  
X-gal (1:500 of 40mg/ml stock)  
IPTG (1:500 of 100mM stock).

LB:

When growing bacteria in LB, we use low salt media:

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

Autoclave, allow to cool & add antibiotics as required (remember, the bacteria carrying your gene after the recombineering are not amp resistant anymore):

Chloramphenicol (1:1000 of 12.5mg/ml stock)  
Amp (1:1000 of 50mg/ml stick)

## ***Appendix 2 – cloning efficiency***

### **The following do effect cloning efficiency:**

#### **Competency of cells**

More competent cells = more correct colonies.

The best way to ensure this is to be gentle to the bacteria, and keep them cold throughout the washing steps.

#### **Size of DNA**

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 problem, but you will get fewer white colonies from a larger insert.

#### **Media/antibiotics**

We've had reports from collaborators that the following can cause recombineering to fail:

Growing the bacteria containing the BACs in high salt (10g/L) LB instead of low salt (5g/L).

Growing the bacteria in high concentrations of Amp (100ug/ml instead of 50ug/ml).

### **The following don't effect cloning efficiency**

#### **Amount of DNA**

In comparisons of 500ng & 10ng of DNA, both gave equal numbers of colonies so don't worry if the PCR doesn't work with great efficiency – its rarely the limiting factor.

#### **Concentration of electrocompetent bacteria**

The volume of ddH<sub>2</sub>O that the bacteria are resuspended in prior to electroporation does not make any difference to the percentage of blue/white colonies. In comparisons bacteria prepared from 25ml competent culture were resuspended in either 50µl or 400µl ddH<sub>2</sub>O, and equal percentages of white/blue colonies were obtained. The advantage of diluting the bacteria more is that you can do more inserts with the same volume of bacteria.