In Vivo Excision of pBSSK- from the λ Uni-ZAP XR vector

Single Clone Excision Protocol

Day 1

Core the plaque of interest from the agar plate and transfer it to a sterile microfuge tube containing 500µl SM buffer and 20µl of chloroform. To do this a obtain a sterile Pasteur pipette, fit the wide end with a bulb, depress the bulb and then stick the narrow end into the plaque of interest, suck it up and then blow it out into the microfuge tube containing the SM (it may be necessary to pipette up and down once or twice to dislodge the agarose plug). Alternatively remove a sterile green tip from a box with a pair of forceps and then grasp it at the dispensing end with your hand (this keeps the end of the green tip relatively sterile) and stick it into the agar and pull out the region containing the plaque. Using a pipette bulb, blow the agar plug out of the green tip into the microfuge tube. Gently mix the plug in the SM by finger vortexing the microfuge tube. Incubate overnight at 4°C (or 1-2h at RT). This phage stock should be stable for 6 months or so.

Streak out E. coli strain XL1-Blue MRF′ onto an LB_{Tet15} plate and E. coli strain SOLR onto an LB_{Kan10} plate.

Pick a single colony of each into separate 5ml of NZY supplemented with maltose (to 0.2% w/v) and grow at 30°C overnight (or at 37°C until OD_{600} = 1.0).

Spin the cells down (use the clinical centrifuge on setting #5 for five to 10 minutes) and decant the spent medium off of the cell pellet.

Resuspend the cell pellet in sterile 10mM $MgSO_4$ using about ½ the volume of culture medium. Determine the OD_{600} of the resuspended pellet. Dilute the cells to OD_{600} = 1.0.

Combine in a sterile culture tube:

200µl of XL1-Blue MRF′ (OD_{600} = 1.0)
250µl of phage stock (the cored plaque) anticipated to be ~1 x 10^5 pfu/ml
1µl of ExAssist helper phage (>1 x 10^6 pfu/ml)

Incubate at 37°C for 15 minutes to allow infection of the E. coli cells (phage will absorb to cells at RT but λ cannot inject DNA until incubated at 37°C).

Add 3ml of LB and incubate at 37°C with shaking for 2.5-3 hours.

Heat the tube to 65-70°C for 20 minutes. This kills the XL1-Blue MRF′ cells.

Spin the tube at ~1000 x g for 15 minutes. (Stratagene recommends 1000 x g but I think full speed on the microfuge is fine.)

Transfer the supernatant into a second sterile tube. This is the excised pBluescript phagemid (**phage**-plas**mid**, a plasmid with an M13 phage origin of replication) encapsulated (packaged) as single stranded DNA into a phage protein particle capable of infecting any E. coli cell that sports an F pilus.

Conceptual synopsis of what is occurring:

The insert was originally cloned into the *Eco*RI and *Xho*I sites of the 41kb Uni-ZAP XR λ insertion vector. A portion of λ sequences flanking the *Eco*RI and *Xho*I sites consists of the 3kb plasmid vector pBluescript SK-. Upon coinfection of the XL1-Blue MRF′ strain with the λ phage and the ExAssist helper phage, the

ExAssist helper phage synthesizes components, which cause the *in vivo* excision of the pBluescript phagemid from the Uni-ZAP XR λ vector. The ExAssist helper phage also provides the phage proteins that encapsulate the ssDNA phagemid DNA. The packaged filamentous phage particles are secreted into the medium and can be purified by centrifugation of the cells and collection of the supernatant. This may then be stored at 4°C for 1-2 months.

To plate the excised phagemids:

> Add 200µl of the freshly grown SOLR cells (as prepared above) to two separate 1.7 ml microfuge tubes.
>
> To one tube add 100µl of the excised pBluescript phagemid
> To the other tube add 10µl of the excised pBluescript phagemid

Incubate at 37°C for 15 minutes to allow infection of the E. coli cells. Both the phagemid and the ExAssist helper phage carried over from the previous in vivo excision step are infectious on the SOLR cells, however the ExAssist helper phage cannot replicate in this strain because it contains an amber mutation (UAG) that requires an amber suppressor tRNA in order to grow.

Plate 200µl of the cell mixture from each microfuge tube onto LB_{Amp150} plates.

Incubate plates overnight at 37°C.

Pick single colonies into LB_{Amp150} medium and grow overnight. Perform minipreps.

Mass Excision Protocol

(Titer the phage library to be excised (see Phage Library Titering) using XL1-Blue MRF′.)

Streak out E. coli strain XL1-Blue MRF′ onto an LB_{Tet15} plate and E. coli strain SOLR onto an LB_{Kan10} plate.

Pick a single colony of each into separate 5ml of NZY supplemented with maltose (to 0.2% w/v) and grow at 30°C overnight (or at 37°C until OD_{600} = 1.0). (No need to include the antibiotics in the overnight cultures.)

Spin the cells down and decant the spent medium off of the cell pellet.

Resuspend the cell pellet in 10mM $MgSO_4$ using about ½ the volume of culture medium. Determine the OD_{600} of the resuspended pellet. Dilute the cells to OD_{600} = 1.0.

Combine in a sterile culture tube a portion of the amplified λ bacteriophage library with XL1-Blue MRF′ at an MOI (multiplicity of infection; i.e., ratio) of 1:10 λ phage particles per one cell. Be sure to excise 10X to 100X λ phage over that which was the size of the original library. This helps to insure statistical representation of the excised clones (thus if the original library was 1 x 10^6 recombinants, then 1X 10^7 to 1 x 10^8 phage should be excised). Add ExAssist helper phage at a 10:1 helper phage-to-cells ratio to insure that every cell is co-infected with λ phage and helper phage.

For example, use:

10^7 pfu of the λ phage (which should be 10 X to 100 X the primary library size).
(We do not know the original titer of the Kieber libraries; however 1 x 10^5 to 1 x 10^6 pfu is probably a good estimate.)

10^8 XL1-Blue MRF′ cells (1:10 λ phage-to-cell ratio).
(For XL1-Blue MRF′, an OD_{600} of 0.3 corresponds to 2.5 X 10^8 cells/ml.)

10^9 pfu of ExAssist helper phage (10:1 helper phage-to-cells ratio).

Incubate at 37°C for 15 minutes to allow infection of the E. coli cells (phage will absorb to cells at RT but cannot inject their DNA until incubated at 37°C).

Add 20ml of LB and incubate for 2.5-3hours at 37°C with shaking. It is strongly recommended that incubation times greater than 3 hours be avoided because this may dramatically alter the clonal representation.

Heat the tube to 65-70°C and incubate for 20 minutes.

Spin the tube at ~1000g for 15 minutes.

Decant the supernatant into a second sterile tube. This is the excised pBluescript phagemid packaged as filamentous phage particles (which may be stored at 4°C for 1-2 months).

Titer the excised phagemid using the SOLR cells.

Preparation of DNA from the *en masse* excised phagemids:

Pick a single colony of E. coli strain SOLR into 10ml LB and grow until the OD_{600} is ~1.0

Add a volume of the excised phagemid to the 10ml culture of SOLR cells that roughly corresponds to 100X the original library titer (~10^7 to 10^8 pfu).

Incubate at 37°C for 15 minutes.

Inoculate this 10 ml into 1000ml of LB_{Amp150}.

Continue to grow this culture until the OD_{600} = 1.0

Prepare DNA using Qiagen Maxi Protocol.

Use this DNA as template in PCR with primers to amplify a cDNA for the gene of interest.

Notes:

The Qiagen Maxi protocol recommends using only 100ml of culture volume. Although we are using 1000ml we are not growing for 12-16 hours; rather we are growing only until the OD_{600} is ~1.0.