

BRED: Bacteriophage Recombineering with Electroporated DNA

Introduction

We have developed a system for generating mutations in lytically replicating mycobacteriophages that we have termed BRED: Bacteriophage recombineering with electroporated DNA. This method utilizes recombineering-proficient strains of *Mycobacterium smegmatis* that have elevated recombination frequencies due to the expression of phage-derived proteins. It is described in further detail in Marinelli *et al.* (2008) and van Kessel, Marinelli & Hatfull (2008). The development of the system for the construction of bacterial mutants is described in van Kessel & Hatfull (2007) and (2008).

We have used this methodology for the construction of a number of different types of mutations, including unmarked deletions, point mutations, small insertions, and gene replacements. The protocol outline is simple and is similar for all procedures:

To make a deletion or small insertion, you will need to construct an approximately 200 bp double-stranded (dsDNA) substrate; for deletions, this should contain 100 bp of homology upstream and downstream of the region to be deleted, making sure that that deletion will be in-frame, if necessary. For small insertions, this substrate should contain the sequence to be inserted, flanked by about 100 bp of homology on each end. To make a gene replacement mutant, you will need to construct a linear allelic exchange substrate (AES) that contains the sequence you wish to introduce, flanked by approximately 100 bp of sequence homologous to either end of the region to be replaced. Mutants containing one or more point mutations can be generated using two synthetic complementary oligonucleotides (oligos), typically 70 nucleotides (nt) containing the (centrally located) mutation. All of these substrates are co-transformed in to electrocompetent recombineering cells with the phage DNA, and plaques are screened for the presence of the mutation by PCR.

There are several critical points to note:

1. We recommend that you use a recombineering strain carrying plasmid pJV53 (or a similar plasmid; Figure 1), which replicates in both *Escherichia coli* and mycobacteria, carries a kanamycin-resistance marker, and has mycobacteriophage Che9c genes *60* and *61* under the control of the inducible acetamidase promoter. Expression of the recombineering functions is accomplished by induction with acetamide.
2. The substrate must be a linear piece of DNA, and we typically make these by PCR, as described in the following protocols.
 - a. Optimal recombination frequencies are obtained when using 100-400 ng recombineering substrate per transformation.
3. In mycobacterial recombineering, the frequency of recombination is also dependent on the length of the targeting substrate homology. When making insertions, deletions or gene replacements, 100 bp of homology on either end is sufficient to recover mutants. Phage point mutations have been made with oligos as small as 70 nt; however, in mycobacteria these can be as small as 48 nt, and this may be true of phages as well.
4. One of the main keys to success is to have good competent cells, and therefore, we have included our protocol below, which routinely generates cells that yield 10^6 transformants/ μ g extrachromosomally-replicating plasmid DNA, using 50 ng plasmid DNA per transformation. It is also important to check the competency of the cells using the DNA of the phage to be mutated. For this reason, we do a few test transformations with various amounts of phage DNA (and no substrate); ideally, ~100-300 plaques should be obtained from a transformation with 50-150 ng phage DNA. The amount of phage DNA can be increased to generate more plaques if necessary, but this should be done without adding large volumes of DNA to the transformation (See #5 below).

5. The time constant of the transformation is important, particularly when transforming large pieces of DNA, such as phage genomic DNA. Inadequate washing of the competent cells or the presence of salt in either the phage DNA or the substrate will adversely affect time constants, which is why all DNA used in these experiments should be resuspended in sterile dH₂O. Ideal time constants are in the range of 19-22 ms, but these will decrease as larger volumes of DNA are added to the reaction, likely due the presence of residual salts. As stated above, it is a good idea to test the competency of your cells and determine the time constants obtained when transforming phage DNA alone. However, addition of the dsDNA substrate will always reduce the time constant to some degree. If you find that addition of your substrate is dropping the time constants too low (i.e. < 18 ms), there are a few options:
 - a. Add less of the substrate. We've been able to make deletions when adding as little as 100 ng of substrate DNA; it is just somewhat less efficient.
 - b. Dilute your competent cells. Sometimes low time constants are a result of cells that are too thick. You can try using 80-60 μ l of cells per transformation and bringing this up to 100 μ l with ice cold water.
 - c. Remake the substrate so that it is more concentrated and you can add the same amount of DNA, while adding less total volume.
6. Since the competency of mycobacteria is relatively low, we employ a co-transformation strategy that uses phage DNA to select against non-transformable cells in the population, and we plate the transformations prior to host cell lysis (see Figure 2). This ensures that only cells competent to take up DNA, and thus, the recombineering substrates, will give rise to infectious centers (termed 'primary plaques'). Depending on the method of screening used, ~5-40% of these primary plaques are observed to contain a mixture of mutant and wild-type DNA.
 - a. Most mutations are screened for using PCR primers that lie outside of the mutated region (flanking primers). So, for example, in the case of deletions, these primers will amplify a smaller product if the mutant allele is present.
 - b. More selective primers annealing either to a specific tag sequence (inserted by the substrate), or across the new junction created by the mutation, can also be employed (termed 'Deletion Amplification Detection Assay (DADA)-PCR and described in Marinelli *et al.* 2008). Point mutations can be engineered to insert a unique restriction site, or can be detected using mismatch amplification mutation assay (MAMA)-PCR (Swaminathan *et al.* 2001).
7. Mutant phage are isolated by re-plating one of the primary 'mixed' plaques that appears to contain a high proportion of the mutant allele, and screening the 'secondary' plaques by PCR (see Figure 2). At this stage, if the mutant is viable, a proportion of these will be pure mutant plaques.
 - a. It is also helpful to make, and screen, a lysate from a plate containing approximately 1000-5000 secondary plaques. If the mutation is in an essential gene, the mutant product will no longer be present when this lysate is analyzed by flanking primer PCR.
 - b. Mutants in essential genes, however, can often be isolated by complementation using a strain of *M. smegmatis* expressing a wild-type copy of the mutated gene.

BRED Protocol: Outline and Materials

STEPS:

- 1) Order oligonucleotides (oligos) to make the recombineering substrate, as described below and in further detail in the methods section (see also Figure 1).
 - a. For deletions or small insertions, you will order an ~100 nt oligo with 50 nt of homology upstream and downstream of the target region. You will also need two 75 nt 'Extender Primers', which add 50 bp of homology to either end of the 100-mer and will be used to generate the dsDNA substrate.
 - b. For a gene replacement, you will order two sets of 75 nt primers. The first will PCR amplify the replacement cassette and add 50 bp of homology flanking the target gene on either end. The second set will amplify the product of the first PCR and add an additional 50 bp of flanking homology on either end of the substrate.
 - c. For point mutations, order two complementary PAGE-purified oligos (70-100 nt) containing the (centrally located) point mutation.
- 2) Order two ~25-30 bp 'flanking primers' to screen for the mutant allele, which anneal upstream and downstream of the insertion/deletion.
 - a. For insertions and gene replacements, you may also order a primer that anneals within the introduced region and can be used with one of your flanking primers.
- 3) Make the recombineering substrate by PCR.
 - a. For deletions or insertions, extend the 100-mer oligo by PCR with your extender primers to generate the 200 bp dsDNA recombineering substrate.
 - b. For gene replacements, amplify the replacement cassette by PCR with the first set of primers. Clean up the product, and use this as a template in a PCR reaction with the second set of primers to generate the full-length substrate.
- 4) Prepare electrocompetent cells of the recombineering strain (mc²155:pJV53) and test competency by transforming various different amounts of phage DNA (typically 50 -150 ng).
- 5) Transform electrocompetent cells with phage DNA, using the amount that gave ~100-300 plaques when transformed alone, and 100-500 ng of the deletion substrate.
- 6) Screen primary plaques by PCR. Once plaque containing the mutant has been identified, re-plate and plaque purify.

Materials:

Steps 1-2, & 5 (Oligos and PCR)→

- TE
- PFU polymerase and 10X buffer
- 10 mM stock of total dNTPs (corresponds to 2.5 mM **each** dNTP)
- DMSO
- Agarose/gel box/TBE/Ethidium bromide
- Phage buffer (10 mM Tris-Cl, pH 7.5; 10 mM MgSO₄; 68.5 mM NaCl; 1 mM CaCl₂)
 - a. Add CaCl₂ from a sterile 0.1 M stock solution after autoclaving.

Step 3 (Electrocompetent cell preparation)→

- 7H9 liquid media
- ADC or ADS
- 20% Tween 80
- 0.1 M stock CaCl₂
- Antibiotic **stock** solutions: CB (50 mg/ml), CHX (10 mg/ml), Kan (50 mg/ml)
- 10% glycerol solution, filter sterilized and chilled on ice before use
- 20% succinate, filter sterilized
- 20% acetamide, filter sterilized
- Microcentrifuge tubes

Steps 4-5 (Transformation and screening)→

- A fresh culture of *M. smegmatis* grown to saturation in 7H9 supplemented with 10% ADC, CB (50 ug/ml), CHX (10 ug/ml), and 1 mM CaCl₂
- Microcentrifuge tubes and electroporation cuvettes, chilled on ice
- Electroporator
- 7H9 liquid media
- 0.1 M stock CaCl₂
- Sterile Pasteur pipettes
- MBTA (mycobacterial top agar is 7H9 with 0.7% bacto-agar)
- 7H10 plates, containing 10% ADC, CB (50 ug/ml), CHX (10 ug/ml), and 1 mM CaCl₂
- Materials for PCR listed above

Methods

1) Order oligonucleotides (oligos) to make the recombineering substrate: (see also Figure 1).

- For **deletions**, order an ~100 nt **PAGE-purified** oligo with 50 nt of homology upstream and downstream of region to be deleted, making sure that the deletion will be in-frame if necessary. For small **insertions** order an oligo containing the region to be inserted, located in the middle of the oligo, and ~40-45 nt of flanking homology on either side.
 - For both insertions and deletions, order two 75 nt '**Extender primers**' that will be used to make the dsDNA substrate:
 1. Design Extender 1 such that it matches the first 25 nt of the 100 nt oligo and has 50 nt additional homology upstream of the deletion/insertion on the 5' end of the primer.
 2. Design Extender 2 such that it matches the last 25 nt of the 100 nt oligo and has 50 nt additional homology downstream of the deletion/insertion on the 3' end of the primer. Then order the **REVERSE COMPLEMENT** of this sequence.
 3. These primers should be **PAGE-purified**.
- For **gene replacements**, order two sets of **PAGE-purified** 75 nt primers: design the first set such that it will PCR amplify the replacement cassette and add 50 bp of homology flanking the target gene on each end. The second set of 75-mers should add an additional 50 bp of homology flanking the target gene to the substrate generated by the first set of primers, such that the product of the second reaction has 100 bp of homology flanking the target gene on either end.
 - For the first primer set, Extender 1 should match the first 25 bp of the cassette and have 50 nt additional homology upstream of the target gene on the 5' end. Design Extender 2 so that it matches the last 25 bp of the cassette and has 50 nt additional nt of homology downstream of the target gene on the 3' end of the primer, then order the reverse complement of this sequence.
 - For the second set, Extender 3 should match the first 25 bp of the product of the first PCR and have 50 nt additional homology upstream of the target gene on the 5' end. Design Extender 4 so that it matches the last 25 bp of the product of the first PCR and has 50 nt additional nt of homology downstream of the target gene on the 3' end of the primer, then order the reverse complement.
- To incorporate **point mutations**, order 2 **PAGE-purified** 70 nt oligos. The first should be homologous to the genomic region to be altered and contain the desired point mutation in its center. The second is the reverse complement of the first oligo.
 - Oligos shorter than 70 nt may also work; minimum length is 48 nt for mycobacterial recombineering, but this has not been tested for BRED.

- 2) **Order primers for mutant screening:** should be ~25-30 nt with a melting temperature $\geq 60^\circ\text{C}$.
- Flanking primers should anneal upstream and downstream of deletion, insertion, or replacement in the phage genome and should generate products, such that the mutant product can be **easily distinguished** from the wild-type (i.e. that are not too similar in size) and neither product is too small (i.e. **> 350 bp**).
 - At this time you may also order more selective primers that anneal either to a specific tag sequence (inserted by the substrate), or across the new junction created by the mutation (termed 'Deletion Amplification Detection Assay (DADA)-PCR, described in Marinelli *et al.* 2008). Point mutations can be engineered to insert a unique restriction site, or can be detected using Mismatch Amplification Mutation Assay (MAMA)-PCR (Swaminathan *et al.* 2001).
 - Resuspend flanking primers to **100 μM** in TE buffer (multiply nmoles by 10 and add that amount TE) and dilute 1:9 in dH_2O to make **10 μM** stock for PCR.

3) **PCR to generate 200 bp recombineering substrate for insertions or deletions:**

- Briefly spin down tubes containing the 100 nt oligo and the Extender primers to collect lyophilized DNA and resuspend in TE buffer as described below. Store all resuspended primers at -20°C .
 - Resuspend deletion/insertion oligo to **1 $\mu\text{g}/\mu\text{l}$** in TE buffer.
 1. Example $\rightarrow 0.09 \text{ mg} \times 1000 = 90 \mu\text{g}$, add $90 \mu\text{l}$ for $1 \mu\text{g}/\mu\text{l}$
 - Dilute deletion/insertion oligo 1/50, to make a $20 \text{ ng}/\mu\text{l}$ solution in dH_2O .
 1. EXAMPLE $\rightarrow 49 \mu\text{l} \text{ dH}_2\text{O} + 1 \mu\text{l}$ of oligo ($1 \mu\text{g}/\mu\text{l}$ stock) = $20 \text{ ng}/\mu\text{l}$
 - Resuspend extender primers to **10 μM** in TE buffer.
 1. Simple trick \rightarrow Multiply nmoles by **100** and add that amount of TE.
- Set up 4 PCR reactions for each substrate (Wear gloves!).
 - Add $2 \mu\text{l}$ (40 ng) diluted oligo to four PCR tubes.
 - To each tube add, in this order:
 1. dH_2O - $68 \mu\text{l}$
 2. 10X PFU Buffer- $10 \mu\text{l}$
 3. 10 mM dNTP stock (2.5 mM each)- $10 \mu\text{l}$
 4. DMSO- $5 \mu\text{l}$
 5. Extender primers (10 μM stock)- $2 \mu\text{l}$ each
 6. *Pfu* Polymerase- $1 \mu\text{l}$
 - You may also make a master mix containing enough components for 4 reactions and then aliquot $98 \mu\text{l}$ per tube.
- Run PCR reactions in Thermocycler.
 - **Parameters:**
 - 96°C -5 min
 - 96°C -30 sec; 58°C^* -30 sec; 72°C -45 sec—For **5 cycles**
 - 96°C -30 sec; 62°C -30 sec; 72°C -45 sec—For **25 cycles**

72°C-7 min
4°C

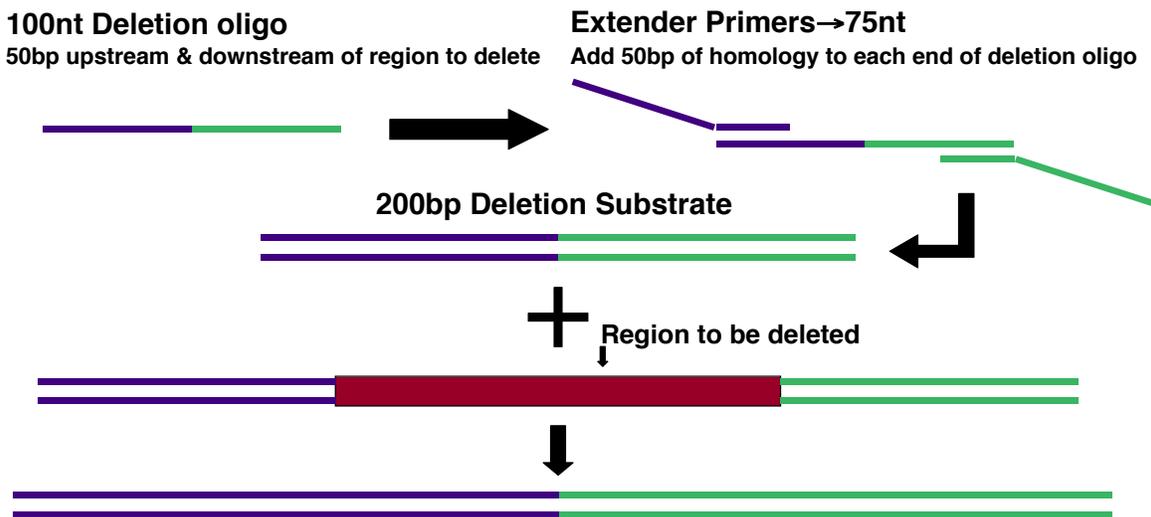
*The annealing temperature for the first 5 cycles is given as 58°C; however, you should check the predicted melting temperature of your Extender primers prior to running the reactions. Check JUST the 25 bp of the extenders that matches the deletion/insertion oligo. If it is lower than 60°C, you should decrease the annealing temperature to 2°C lower than the melting temperature of your Extender that has the lowest melting temperature.

- Check your PCR yield by running ~5-10 µl of each reaction on a 1 – 1.2% agarose gel.
- If product is present, clean-up reactions with QIAquick PCR Purification Kit (QIAGEN) or MinElute Reaction Clean-up Kit (QIAGEN).
 - Reactions that worked should be pooled—i.e. run all reactions for the same substrate over **one** QIAquick column.
 - Resuspend cleaned reactions in 30 µl sterile **dH₂O**.
- Quantify your cleaned substrate, either by running on an agarose gel with a quantitative ladder or by some other means (e.g. Nano-drop).
 - Ideal concentration is ~100 ng/µl or greater→you want to be able to add a significant amount of the substrate (~200-400 ng) to your transformation without adding too much volume to the reaction.

**To construct a gene replacement, resuspend all primers in TE buffer to 10 µM. Perform the first round of PCR as described above, using ~20 ng of the DNA containing the replacement cassette as a template per reaction. Check, pool and cleanup the PCR amplification with QIAGEN QIAquick columns as described above, although it may be necessary to gel extract if there are non-specific products. Quantify the purified product, and perform as second round of PCR with the second set of extender primers, using the product of the first reaction (~50-100 ng) as a template.

***When creating a point mutation, resuspend the oligos to 1 µg/µl and dilute to 200 ng/µl in dH₂O for transformations; transform 1 µl of each oligo. These do not have to be annealed prior to transformation.

Figure 1: Construction of a deletion substrate by PCR.



4) Preparation of recombineering strain stocks and electrocompetent cell preparation of the recombineering strain → mc²155:pJV53:

- Transform plasmid pJV53 into *E. coli* DH5 α for plasmid preparation and maintenance, selecting for kanamycin resistant transformants.
- Mini-prep the plasmid, and transform pJV53 DNA into electrocompetent *M. smegmatis* cells* by electroporation, selecting for kanamycin resistant transformants.
- Grow up a small culture of the recombineering strain (mc²155:pJV53) in 7H9 medium with 10% ADC, 0.05% Tween 80 and KAN (20 μ g/ml), and freeze down aliquots of this strain in 20% glycerol for future use.
- Prepare electrocompetent cells as described below:
 - It is **VERY IMPORTANT** that the cells are prepared on ice, centrifuged at 4°C, and the 10% glycerol solution is **ice-cold**. Any volume of cells can be prepared; 100 ml yields approximately 40 aliquots of competent cells.
- Grow a ~3 ml culture of the recombineering strain (pJV53) in 7H9 medium with 10% ADC, 0.05% Tween 80 and KAN (20 μ g/ml) to saturation (~2 days).
- Inoculate 100 ml 7H9 induction medium (7H9/KAN/Tween/0.2% succinate/1 mM CaCl₂) to an OD₆₀₀ ~0.02 and incubate shaking overnight @ 37°C (**No ADC**).
 - You may inoculate 1 or 2 different ODs to ensure cultures do not overgrow.
- When culture reaches OD₆₀₀ of ~0.4, add acetamide to a final concentration of 0.2% and incubate shaking @ 37°C for **3 hr**.
- Divide culture into 4 sterile tubes (25 ml each) and place cells on ice for 30 min–2 hr.
- Centrifuge and pellet cells at 5000 RPM for 10 min.
- Wash cells with 1/2 vol. (~12.5 ml per tube, 50 ml total) 10% sterile ice-cold glycerol.
 - Pipette up and down until the clumps of cells are dispersed.
- Pellet cells as before; wash with 1/2 vol. (~12.5 ml per tube, 50 ml total) 10% sterile ice-cold glycerol. Combine cells into 2 tubes (~25 ml each).
- Pellet cells as before; wash with 1/4 vol. (~12.5 ml per tube, ~25 ml total) 10% sterile ice-cold glycerol.
- Pellet cells as before; resuspend in ~1/20 vol (4-5 ml **total**) 10% sterile ice-cold glycerol.
 - The cells should not be thick or yellowish, but they should not be too dilute either. If unsure, error on the side of 'too thick' as they can be diluted later.
 - If the time-constants from the transformation are consistently low (<18 ms), this is an indication that the cells are too thick.
 - If the cells are too thin, the time constants will be good (19-22 ms), but the number of plaques obtained will be low (<100 plaques from 150 ng phage DNA).
- Aliquot 110 μ l cells into chilled 1.5 ml microcentrifuge tubes.
 - Cells can be stored in larger aliquots (e.g. 220 μ l = 2 rxns or 440 μ l = 4 rxns per tube) and split prior to the addition of DNA and electroporation.
 - Freeze on dry ice and store at -80°C; thaw on ice when ready to use.

*To make electrocompetent cells of **WT** *M. smegmatis*, grow cells in standard media (with ADC & Tween) to an OD₆₀₀ of 0.8-1, and follow protocol outlined above, omitting the induction step.

5) Transformation (See also Figure 2):

- Place tubes of cells on ice, ~100 µl cells per transformation and thaw for ~10 minutes.
 - If frozen aliquots contain cells for multiple reactions, chill 1 fresh microcentrifuge tube for each transformation, and aliquot 100 µl per tube.
- While (or before) cells are thawing, add 900 µl 7H9 / 10% ADC / 1 mM CaCl₂ to sterile test tubes (one tube per transformation) and label.
- Pipette DNA into cells and mix gently.
 - Add 50-150 ng of phage DNA.
 - Add 100-400 ng of 200 bp deletion substrate—no more than ~5 µl.
- Incubate on ice for ~10 min.
- Pipette cells and DNA into chilled cuvette.
- Wipe off cuvette well, and place in electroporator holder.
- Electroporate (Set to 2.5 kV, 1000 Ω, 25 µF).
 - Push both buttons simultaneously and hold until beeping sound is heard.
 - Check time constant→should be >18 ms, and 19-21 ms is best. Time constants <18 ms will result in very few to no plaques.
 - If the machine arcs, you will hear a popping sound, and cells are likely dead. This transformation must be repeated.
- Using a Pasteur pipette, carefully remove some of the 900 µl of media from one of the test tubes and add this to the cells in the cuvette; then pipette all of this back into the test tube.
- Incubate at 37°C for 30 min - 2 hr shaking at 250 rpm (Don't leave longer as cells will lyse).
- Remove one 7H10 plate (ADC/CaCl₂/CB/CHX) for each transformation from the refrigerator, warm to room temperature and label appropriately
- Prepare **plating mix**:
 - ~5 minutes before recovery will be ready, carefully melt a bottle of MBTA.
 - When cells are fully recovered, mix 1.5 ml 7H9, 50 µl 0.1 M CaCl₂ (Final conc. 1 mM) and 2.5 ml molten MBTA **per plate** (in appropriately sized sterile tube or flask) and cool a few seconds.
 - To this mix, add ~250-300 µl fresh *M. smegmatis* culture (no Tween) **per plate**.

NOTE: amounts given are for 1 plate; this should be scaled up when you are plating multiple recoveries at the same time

- To make lawns:
 - Using 5 ml pipette, transfer 4 ml plating mix to the tube containing your electroporation recovery.
 - Carefully pour a thin layer onto the surface of a 7H10 plate, gently swirl to distribute evenly.

- Incubate plates overnight (~24-36 hr) at 37°C.

6) Screen plaques (see also Figure 2):

- Pick individual plaques into 100 µl phage buffer; make sure CaCl₂ is added to 1 mM.
 - Pick ~18-25 initially; this is often enough to find positives, but in some cases, you may need to check more.
 - Incubate at room temperature for 1-2 hr or at 4°C overnight; store plaques at 4°C.
 - Aliquot 1 µl of each plaque to use as a template for PCR with the flanking primers.
 - Always set up a control reaction with WT phage DNA (5-10 ng).
 - Mutant products that are larger than the WT product (such as insertions) may be difficult to detect with flanking primers, and their identification may require the use of a primer specific to the mutant allele.
 - Make a master mix containing the following components (per reaction), and set up enough for at least one extra reaction; aliquot 19 µl master mix per tube:
 1. dH₂O-12.5 µl
 2. 10X PFU Buffer-2 µl
 3. 10 mM dNTP stock (2.5 mM **each** dNTP)-2 µl
 4. DMSO-1 µl
 5. Extender primers (10 µM stock)-0.5 µl each
 6. PFU Polymerase-0.5 µl
 - Run PCR reactions in Thermocycler:
 - **Parameters:**
 96°C-5 min
 96°C-1 min; X°C*-1 min; 72°C-1.5 min per kb of product—**30 cycles**
 72°C-7 min
 4°C-∞
- *2° lower than the melting temperature of the primer with the lowest melting temp.
- Run PCR reactions on an agarose gel.
 - Positive 'mixed' plaques will have a faint mutant band of the expected size in addition to the wild-type band.
 - Mutant band can be difficult to see; so you may have to increase UV intensity.
 - Once mixed plaques containing the deletion are identified:
 - Serially dilute plaque (10⁻³, 10⁻⁴, and 10⁻⁵) in phage buffer, and infect ~300 µl fresh *M. smegmatis* (no Tween) with 10 µl of each dilution in sterile tubes.

- Adsorb at room temp for ~30' and make top agar overlays→ In sterile vessel combine ~2.5 ml molten MBTA + ~2.5 ml 7H9 + 50 µl CaCl₂ **per plate**. Add ~4-5 ml to each tube and plate on 7H10/CB/CHX.
- Pick at least 18 individual 'secondary' plaques from plate(s) with isolated plaques, and make a lysate from the plate containing ~1000 plaques (typically 10³ plate).
 - Lysates are made by flooding plate with 3-5 ml phage buffer (with 1 mM CaCl₂), allowing this to stand for at least 1 hr, then collecting the buffer in a sterile tube.
- Perform PCR with flanking primers as before on 1 µl of each purified plaque and 1 µl of the lysate.
 - If the mutant is viable, a number of these will contain only the mutant product, indicating that this plaque is a pure mutant.
 - If the deletion band can no longer be visualized in the lysate PCR, the mutant is likely not viable.
 - If the mutant product is present in the lysate but all of the secondary plaques are WT, you may need to pick more individual plaques to find a pure mutant.
 - For difficult to isolate mutants, it may be helpful to pick pools of 5-10 secondary plaques at a time (in at least 250 µl of buffer), thus allowing you to screen more plaques at once. When a pool containing the mutant is found, it can be re-plated and again, individual plaques should be screened by PCR.
- Once a pure mutant plaque is identified, re-plate this, and make a lysate from a plate containing a good pattern of webbing. This lysate should be filter sterilized and confirmed to contain only mutant phage by PCR.
 - In this case, use at least 5 ml of buffer, and make sure to collect as much as possible from the plate.

Figure 2: Construction of a gene deletion using BRED.

