Chapter 5

Appendix

5.1 Cloning

Cloning is the process to ligate fragments of DNA. There are many methods such as restriction enzymes, PCR, SLIC, and Gibson. Below is an overview of the cloning strategies used in most of this study.

For most seamless cloning methods, the ends of the strands of DNA need some amount of homology to drive their association and subsequent ligation. One strategy to create this homology is to use PCR. Since the primer is incorporated into the amplified DNA, one can specify the nucleotide sequences at the ends of an amplified dsDNA by designing primers.

5.1.1 Primer design

The specificity of a PCR primer is often approximated by the melting temperature. The higher the melting temperature of a primer, the tighter the primer will bind to that specific sequence. PCR regulates the annealing of DNA (i.e. primers) by controlling the temperature of the reaction.

Primer annealing depends on the GC content of the primer and on the reaction

conditions. Guanine and cytosine each associate with three hydrogen bonds while adenosine and thymine associate with two hydrogen bonds. As a result, a nucleotide sequence with a high GC content (above 60%) will generally have a higher melting temperature than a nucleotide sequence with a low GC content (below 30%). Primer annealing is also affected by the concentration of the solutes such as the concentration of the primers themselves, the dNTPs, and the Mg^{2+} concentration.

When designing a primer, the specificity of binding is approximated by the melting temperature(Tm). The Tm can be calculated by a variety of software packages take into account factors such as GC content, dNTP concentration, primer concentration, and Mg^{2+} concentration. However, the existence of multiple Tm calculators that report different predicted melting temperatures suggests there is not good agreement on the method to calculate a primer's melting temperature. **It is imperative that you use a Tm calculator that at the very least takes into account the buffer salts and primer concentrations**.

In my work, two factors heavily influenced the predicted primer melting temperature. I have had good success using these the Tms reported by calculated by 'Netprimer', by PremierBiosoft, and 'Oligo Analyzer', by IDT.

In general I design annealing regions of my primers to have a Tm around 65°C. This strategy has usually been a good balance between specificity and an annealing region of reasonable length. The term 'annealing region' is used to emphasize the fact that depending on the cloning strategy, different regions of the primer may be designed to anneal to different sequences and the Tms should be calculated independently of one another.

5.1.2 Site directed mutagenesis

Site directed mutagenesis an efficient strategy for implementing mutations in a plasmid. In the simplest case the strategy can modify or insert a few nucleotides.

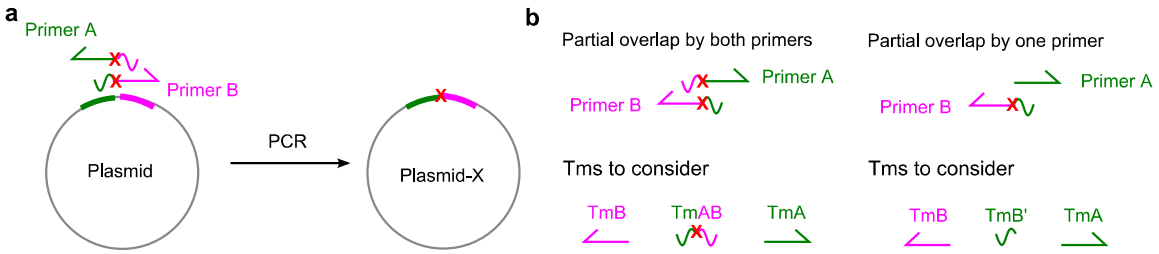

Figure 5.1: General outline for site directed mutagenesis. (**a**) Site directed mutagenesis is a strategy for amplifying circular DNA and more interestingly, integrating an arbitrary nucleotide sequence into a plasmid. (**b**) When designing primers for site directed mutagenesis, one needs to design three Tms for for three annealing interactions. Two annealing interactions are needed to drive the 3' end of each primer to bind to the template. A third annealing interaction of the 5' ends associating with each needs to be considered as well. Both primers can anneal to each other or one primer can be designed to exclusively anneal to the other. Note in the latter case, the mutation is not considered in the Tm calculation since the mutation does not exist on the other primer. In either situation, **some portion of the 3' end of each primer should exclusively anneal to the template for efficient annealing to the template.**

In more complicated cases a sequence of arbitrary size can be incorporated into any plasmid.

The basic idea is that since a PCR primer is incorporated into the dsDNA final product of a PCR, any nucleotide modifications in the PCR primers will be incorporated into the final sample of dsDNA. Further, by including significant homology in the 5' ends of the primers, the primers will prime each other to facilitate ligation of the ends of a strand of dsDNA. If only two primers are used on a plasmid then the primers will circularize the product to create a plasmid containing the mutation of interest.

The biggest concern in the design of site directed mutagenesis primers is that for the reaction to occur efficiently, the primers need to preferentially bind to the template rather than themselves in spite of their 5' homology. This can be addressed by purposely designing the primers such that the 5' region anneals only to the other primer while the 3' end anneals only to the template.

To design primers for simple site directed mutagenesis, one needs to consider three

annealing events and as a result, one needs to design for three Tms. The 3' end of each primer need to anneal to the template strand for amplification. Then the 5' end needs to anneal to each other. This Tm can be designed as a hybrid sequence whereby the nucleotide sequence on the 5' end of both primers can be considered as one nucleotide sequence. The 3rd Tm is for this overlap sequence.

The mutagenesis PCR reaction itself is a standard PCR with high fidelity polymerase to reduce incorporation of point mutation due to the fidelity of the polymerase. At the end fo the PCR, one would expect a running at the length of fully linearized DNA. DPNI digest post mutagenesis is usually standard.

In my work I typically use phusion(NEB) in HF buffer with 5% DMSO but any PCR strategy using a HF DNA polymerase should be sufficient.

Reagents for site directed mutagenesis with phusion DNA polymerase

**Based off of default PCR protocol in NEB phusion manual*

1. 10 mM dNTPs
2. 10uM solution of each primer
3. Sample of template
4. DMSO
5. HF buffer
6. DI water
7. Phusion DNA Polymerase (NEB)
8. DPNI
9. Gel electrophoresis setup

PCR mix for site directed mutagenesis with phusion DNA polymerase

1. 35uL DI water
2. 2.5 uL DMSO
3. 10uL HF buffer
4. 1uL dNTP mix
5. 0.5uL of each primer
6. 0.5uL of template
7. 0.5uL phusion DNA polymerase

PCR protocol for site directed mutagenesis with phusion DNA polymerase

1. See default Phusion DNA Polyerase Protocol 5.3.7

Typical workflow for site directed mutagenesis with phusion DNA polymerase

1. 1 - Do site directed Mutagenesis PCR
2. 2 - Confirm PCR via gel electrophoresis
3. 3 - If product is confirmed, add 1uL DPNI to PCR mix, incubate at 37°C for 1hr and then heat kill
4. 4 - transform 10uL of PCR into chemically competent cells, plating entire outgrowth

5.1.3 Ligating dsDNA with PCR

One strategy to ligate dsDNA with PCR. One can imagine dsDNA as a really long primer with a partner strand that completely complements it. As it turns out, one can reasonably expect ends of dsDNA to behave as a PCR primer in a PCR reaction. By setting up a PCR reaction with dsDNA that are designed to anneal completely or in a hybrid fashion, each strand of dsDNA will be incorporated on the end of the other much in the same fashion as a primer is incorporated into the final dsDNA. One can either ligate strands of dsDNA to create a hybrid strand of dsDNA or, if the ends of the final strand of dsDNA anneal to each other, create a plasmid.

In fact, just using dsDNA fragments without any primers in a PCR has been previously described as Circular Polymerase Extension Cloning (CPEC) [75]. However, optimizing CPEC reactions may be tricky. I typically use primers to drive the amplification of circular DNA, in essence doing site directed mutagenesis to create circular DNA where the 'mutation' is already on the template strand of DNA.

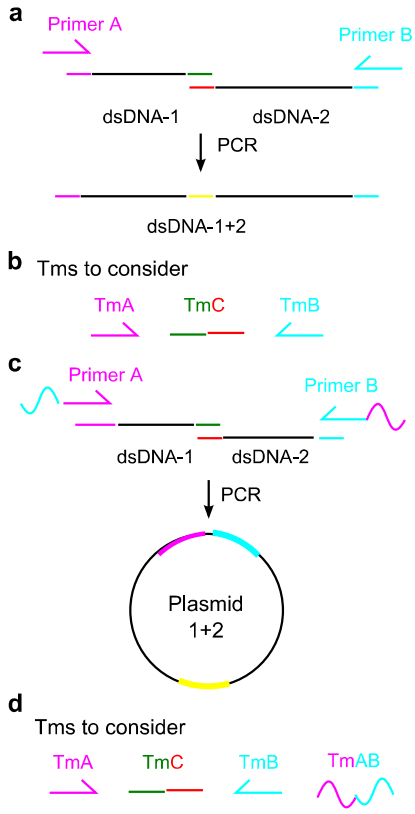

Figure 5.2: (**a**) PCR can also be used to ligate strands of dsDNA (**b**) When considering ligation via PCR three Tms need to be accounted for. The Tms for the primers that bind to either end of the final strand, and the Tm of the hybrid sequence at the interface of the two strands. (**c**) By designing additional overlaps in the 5' end of the primer, this can be extended to creating a circular piece of DNA. (**d**) In this case, four Tms needs to be considered. The Tms of the 3' ends of the primers annealing to the dsDNA, the dsDNA that anneal together, and the 5' ends of the primers.

Reagents for ligation of dsDNA with PCR

1. 10 mM dNTPs

2. 10uM solution of each primer that amplify ligated dsDNA

3. samples of each dsDNA species to be ligated
4. DMSO
5. HF buffer
6. DI water
7. Phusion DNA Polymerase
8. Gel electrophoresis setup

PCR mix protocol for ligation of dsDNA with PCR

PCR Mix Protocol

1. 35uL DI water
2. 2.5 uL DMSO
3. 10uL HF buffer
4. 1uL dNTP mix
5. 0.5uL of each primer
6. 0.5uL of each dsDNA sample
7. 0.5uL phusion DNA polymerase

PCR protocol for ligation of dsDNA with PCR

1. See default Phusion DNA Polymerase Protocol 5.3.7

Typical workflow for ligation of dsDNA with PCR

1. 1 - PCR with phusion
2. 2 - Confirm PCR via gel electrophoresis

5.1.4 Sequence and Ligation Independent Cloning (SLIC)

Originally described by Li and Elledge [53].

Cloning by SLIC is an economical strategy for ligating dsDNA. dsDNA is first treated with T4 DNA polymerase which has a 3' exonuclease domain. This creates sticky ends on either end of dsDNA. The reaction is then quenched with the addition of a single nucleotide triphosphate. dsDNA with sticky ends are then mixed together, allowing the sticky ends with significant homology to bind together. This mix is then transformed into *E. coli* where it is assumed DNA repair machinery repairs the nicks and gaps in DNA mixture are repaired to make a functional plasmid.

Below is my working protocol. There is significant room for optimization on a case by case basis, but the protocol below has been sufficient for most of my cloning. Most of my dsDNA samples have regions of homology with a predicted melting temperature around 65°C.

SLIC is prefect for ligating two strands of dsDNA together. In such a reaction I would expect about 50-200 colonies on the final plate. One strategy for getting SLIC cloning to work on multiple fragments of DNA is to ligate some of the fragments beforehand via PCR and then using SLIC to create the final plasmid as SLIC is typically faster (45 min)than doing PCR ligation.

SLIC cloning protocol

Reagents

1. 10 mM dGTP - Can be any other dNTP
2. T4 ligation buffer
3. NEB buffer 2.1 (i.e. NEB Buffer 2 with BSA)
4. At least 18 uL of spin column purified dsDNA
5. BSA

6. T4 DNA Polymerase

SLIC cloning reaction Protocol

1. For each dsDNA species mix 18 uL of dsDNA with 2uL NEB buffer 2.1 in a fresh eppendorf tube
2. Add 0.5 uL of T4 DNA Polymerase
3. Incubate at 25°C for 15 min
4. Add 2uL of dGTP to reaction, put on ice

   Note: dsDNA treated with T4 DNA polymerase can be stored at -20° for about a month

   according to the relative size of fragments
5. Mix equimolar amounts of treated dsDNA in a final reaction volume of 9 uL

   Note: I usually assume each sample is at roughly the same mass concentration and scale amounts

   according to the relative size of fragments
6. Add 1 uL T4 buffer
7. Incubate mix at 37°C for 30min
8. Transform entire mix into competent cells - I usually transform into chemically competent cells.

5.1.5 Isothermal Assembly (ITA/Gibson)

Originally described by Gibson et al [34].

Gibson is basically SLIC with an order of magnitude increase in efficiency. This has been overkill for two fragment ligations as if I used Gibson cloning to ligate two

strands of dsDNA, I would typically get a lawn of growth on the plate the next day. Gibson is perfect for one pot ligation of multiple strands of dsDNA.

Gibson/ITA cloning protocol

Reagents *See section 5.2.4 for buffer recipes

- Gibson Master Mix
- 4X DNA mix for Gibson
- chemically competent cells

Gibson cloning rxn

1. 15 uL 1.33 x master mix
2. 5 uL 4x DNA mix
3. Incubate at 50°CC for 15-60 min
4. Transform 10uL of rxn into chemically competent cells, plate entire volume

5.1.6 Restriction Enzyme Cloning

Although predating overlap based cloning, restriction enzyme cloning is still a valuable strategy for cloning. Since restriction are specific to their restriction site, they can facilitate the modular ligation of dsDNA. The one caveat to their use is that one must take care to watch out for unwanted restriction sites in their constructs. TypeII restriction enzymes such as BsaI have expanded the utility of restriction enzyme cloning. TypeII restriction enzymes cut some distance away from their binding site. Arbitrary cut sites can be designed depending on the positioning of the binding site. This enables the one-pot cutting and ligation as described by Engler *et al.* [30]

Homology based cloning is great until constructs have significant regions of homology within themselves. As a result, when isolating or ligating fragments via PCR,

significant mis-annealing mis-amplification may occur. One strategy around those issues is to use restriction enzymes to isolate and ligate sequences of interest.

One can also use restriction enzymes and overlap based cloning together. Rather than use PCR to isolate strands of dsDNA, dsDNA prepared with a restriction enzyme can also be used. This may be useful in generalizing the use of a particular fragment such as the backbone of an expression vector where one wants to easily insert a variety of species of dsDNA.

5.1.7 Colony PCR screening of clones

After transforming a clonine project and on observing colonies on a plate, it is necessary to screen the colonies that contain the correct construct. One convenient strategy is to do colony PCR to screen for the insert in the presence of the backbone. This can be done directly from the plate and can save some effort in screening colonies.

Since the precise sequence is not important, a high fidelity polymerase is not necessary. Colony PCR is not a definitive assay, it is more suited for distinguishing different species of plasmid by the banding pattern that come out of the reaction. More precise characterizations may or may not be possible. As a result, after colony PCR it is often necessary to further screen plasmids each species of plasmid that is detected by colony PCR with either a test digest or simply checking the size of the uncut plasmid.

When selecting primers for colony PCR one can use a primer that binds to the gene of interest and another that binds to the backbone. However, if one is certain the insert will not be background, primers that exclusively amplify the insert are sufficient.

Reagents for Colony PCR with Taq DNA polymerase

1. PCR strip tubes - Scale as necessary, I usually use one strip of 8 PCR

tubes for convenience

2. Standard Taq reaction buffer
3. 10mM dNTP stock
4. PCR primers
5. LB media
6. Sterile culture tubes

Protocol for colony PCR

1. Aliquot 5uL of LB into each PCR tube
2. Aliquot 5mL selective LB media into culture tube
3. Pick a colony and resuspend in the 5uL media in PCR tube
4. Transfer about 3.5uL of resuspension to 5mL selective media
5. Prepare a **Taq PCR mastermix** Note: I typically scale for 25uL PCR reactions

   Taq PCR mastermix for 8, 25uL reactions with some leftover for pipetting error

   (a) 191.5 uL H_20
   (b) 22.5 uL Taq standard buffer
   (c) 4.5 uL 10mM dNTP
   (d) 2.5 uL of each 10 uM PCR primer stock
   (e) 2.5 uL of Taq DNA Polymerase
6. Aliquot about 25uL per PCR tube
7. Run PCR on themocycler default Taq Protocol 5.3.8
8. Move culture tubes to incubator shaker.
9. Run about 10uL of PCR on a gel, discard unwanted culture tubes.

5.1.8 DNA synthesis strategies

Summarized in Wu et al [110].

It sometimes becomes necessary to synthesize a novel nucleotide sequence. One strategy is to incorporate the novel sequence on the ends of a preexisting strand of dsDNA. Another strategy is to completely synthesize the sequence from a set of primers.

To add novel sequence to the end of dsDNA, one strategy is to use multiple primers where the 5' of each primer iteratively builds the desired sequence. To design primers using this strategy, one needs design the 3' Tm to anneal of each to the 5' of the primer(or template dsDNA) downstream of it. If circularized DNA is desired, then the 5' of the upstream most primer on both ends of the PCR need to be designed to anneal to each other like in site directed mutagenesis. When doing the PCR the primers not at the ends of the PCR need to be 10 times more dilute than the standard working concentration of primers in a PCR. This helps ensure the final dsDNA product will be amplifications of the outer most primers and as a result, contains the synthetic sequence in its entirety.

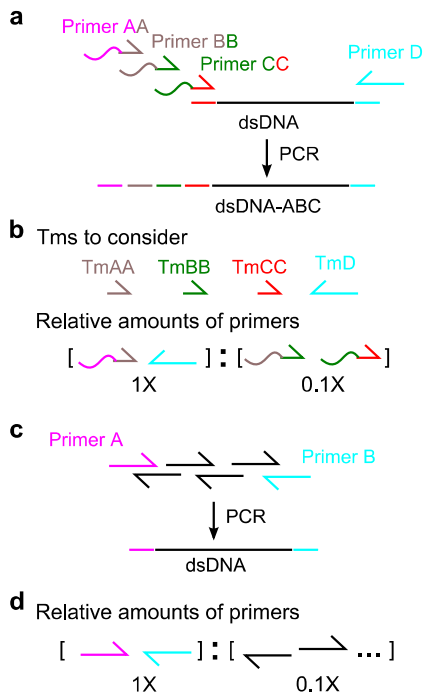

Figure 5.3: (**a**) PCR can also be used to ligate strands of dsDNA (**b**) When considering ligation via PCR three Tms need to be accounted for. The Tms for the primers that bind to either end of the final strand, and the Tm of the hybrid sequence at the interface of the two strands. (**c**) By designing additional overlaps in the 5' end of the primer, this can be extended to creating a circular piece of DNA. (**d**) In this case, four Tms needs to be considered. The Tms of the 3' ends of the primers annealing to the dsDNA, the dsDNA that anneal together, and the 5' ends of the primers.

Another strategy for synthesizing dsDNA over 80bp (the size cutoff of what a typical spin column will bind to) is to use a sequences of primers that overlap each

other in the opposite direction. Although convoluted, fortunately the design of these primers has been automated in the software package DNAworks which is available for free (http://helixweb.nih.gov/dnaworks/). Simply input in your desired nucleotide sequence with some constraints and the algorithm will design a set of primers optimized for PCR based synthesis. I typically request primers with an annealing temperature between 62 and 65°C between 45 and 50nt long. When putting together the PCR the primer at the end of the sequence are at the standard PCR working concentration while all the primers in the middle are 10 fold dilute. Again, this helps to ensure the final dsDNA product will be amplifications of the outer most primers and as a result, contains the entire synthetic sequence.

The above two strategies can be mixed and matched depending on the situation.

PCR DNA synthesis with dsDNA as a template protocol

Reagents (For PCR with Phusion(NEB) high fidelity polymerase with HF buffer)

1. 10 mM dNTPs
2. 10uM solution of each primer at the end of the final construct
3. 1uM solution of primers that are in the middle of the construct
4. sample of template dsDNA
5. DMSO
6. phusion HF buffer
7. DI water
8. Phusion DNA Polymerase
9. Gel electrophoresis setup

PCR Mix Protocol

1. 35uL DI water

2. 2.5 uL DMSO
3. 10uL HF buffer
4. 1uL dNTP mix
5. 0.5uL of each 10uM solution of the two primers at the end
6. 0.5uL of each 1uM solution of primers in the middle
7. 0.5uL of each dsDNA sample
8. 0.5uL phusion DNA polymerase

PCR protocol

1. See default Phusion DNA Polyerase Protocol 5.3.7

Typical Workflow

1. 1 - PCR with phusion
2. 2 - Confirm PCR via gel electrophoresis

PCR DNA synthesis with only primers protocol

Reagents (For PCR with Phusion(NEB) high fidelity polymerase with HF buffer)

1. 10 mM dNTPs
2. 10uM solution of each primer at the end of the final construct
3. 1uM solution of primers that are in the middle of the construct
4. DMSO
5. HF buffer
6. DI water
7. Phusion DNA Polymerase

8. Gel electrophoresis setup

PCR Mix Protocol

1. 35uL DI water
2. 2.5 uL DMSO
3. 10uL HF buffer
4. 1uL dNTP mix
5. 0.5uL of each 10uM solution of the two primers at the end
6. 0.5uL of each 1uM solution of primers in the middle
7. 0.5uL phusion DNA polymerase

PCR protocol

1. See default Phusion DNA Polyerase Protocol 5.3.7

Typical Workflow

1. 1 - PCR with phusion
2. 2 - Confirm PCR via gel electrophoresis

5.2 Buffer/Media Recipes

5.2.1 10X TSS transformation buffer

Buffer composition (in LB)

- 10% PEG 8000
- 5% DMSO v/v
- 30mM MgCl2

- pH 6.5

Reagent list

- 0.2 uM syringe filter
- 60 mL sterile syringe
- 2 sterile 50 mL conical tube
- 5.263 g PEG 8000
- 2.5mL DMSO
- 50mL LB media
- 0.321 g MgCl2 hexahydrate (203.31 g/Mol)

Protocol (super precise version due to reports DMSO degrades polyethersulfone filter membrances)

1. Dissolve PEG8000 and MgCl2 in 50mL LB
2. pH to 6.5. Make sure to mix well, solution is a bit viscous
3. Filter sterilize solution into a sterile container
4. Transfer 47.5 mL to another clean container
5. add 2.5 mL DMSO
6. Done! store 10X TSS at 4°C

5.2.2 5X KCM transformation booster

Buffer composition (in H_2O)

- 0.5 M KCl
- 0.15 M CaCl2
- 0.25 M MgCl2
- Store at -20°C

5.2.3 Modified MOPS minimal media

Modified MOPS minimal media composition

1. MOPS
2. Glycerol -
3. Casein Amino Acids
4. Sodium Bicarbonate
5. Potassium phosphate
6. Thiamine

5.2.4 Gibson Mixes

Buffer recipes and protocol based off Gibseon *et al.* [34]. Working protocol developed by Dr. Joff Silberg.

Reagents

- 1 M Tris Hcl pH 7.5 (20 mL from Shirley)
- 2 M MgCl2 (10 mL from Shirley)
- 100 mM stocks of each dNTP (dA, dC, dG, dT from Roche)
- 1 M dithiothreitol (DTT freezer stock)
- 50 mM NAD+ (0.2 mL, oxidized from NEB)
- PEG-8000 (solid chemical shelf)
- T5 Exonuclease and Buffer (Epicentre; 1:50 dilution in water/Tris, 10 U/uL stock)
- Phusion DNA Polymerase 2 U/uL (NEB; no dilution)

- Taq Ligase 40 U/uL (NEB; no dilution)

5x Gibson rxn buffer

1mL mastermix of dNTP and buffer components, enough for 8 rxn buffer aliquots. 1 5X Gibson rxn buffer aliquot yields 250 reactions.

1. 500 uL 1M Tris HCl pH 7.5
2. 25 uL 2M MgCl2
3. 10 uL 100mM dATP
4. 10 L 100mM dCTP
5. 10 L 100mM dGTP
6. 10 L 100mM dTTP
7. 50 uL 1 M DTT
8. 250 mg PEG-8000
9. 100 uL 50mM NAD+
10. water to 1mL
11. Make 8 aliquots of 120uL

50X Dilute T5 Exo Stock

V_{tot} = 100 uL, for 10 master mixes, 250 rxns per master mix.

1. 2 uL 10 U/uL T5 exo
2. 1 L 1M Tris 7.5
3. 97 L water

Gibson Master Mix Recipe

1.33X, used for final reaction. V_{tot} = 375 uL, enough for 25 reactions.

1. 100 uL 5x Gibson rxn buffer buffer (from above)
2. 10 uL 50X Dilute T5 Exo Stock
3. 6.25 uL Phusion pol
4. 50 uL Taq Ligase (whole tube from NEB)
5. 208.75 uL Water
6. Makes 25 aliquots of 15 uL

4x DNA mix for Gibson cloning

V_{tet}= 5 uL, for 1 reaction. DNA samples should be equimolar

1. 1 uL DNA 1 (2-20 ng/uL/kb)
2. 1 uL DNA 2 (2-20 ng/uL/kb)
3. 3 uL water

5.3 General Protocols

5.3.1 Red λ mediated genome modification in *E. coli*

Originally described by Datsenko and Wanner [23]. Further formalized by Sharan *et al.* in [87]. Protocol below is adapted from above

Further online resources and plasmids provided by the Court lab, http://redrecombineering.ncifcrf.gov/Home.html

, and openwetware,
http://openwetware.org/wiki/Recombineering/Lambda_red-mediated_gene_replacement.

The Keio collection by Baba *et al.* contains a useful library of primers to simple KO most genes in *E. coli* [4].

Reagents

- Host strain with pSIM29
- dsDNA containing flt flanked antibiotic resistance cassette (recommend CmR or KanR)

5.3.2 Integrase mediated genome modification in *E. coli*

Additional integration sites and protocol refinements described by St-Pierre *et al.* [97]. Those protocols were not used in this study as integration of pzs4-Lac/Tet derivatives into the attb site of *E. coli* was sufficient.

Reagents

- Host strain with pSIM29
- dsDNA containing flt flanked antibiotic resistance cassette (recommend CmR or KanR)

5.3.3 Preparation of chemically competent cells

TSS chemically competent cells are competent cells prepared with 10X TSS transformation buffer. Transformation mixtures typically uses the additive 5X KCM to boost transformation efficiency. TSS buffer was originally described by Chung *et al.* and has since been referenced by online lab websites such as openwetware [19].

TSS chemically competent cells provide a good balance between ease of use and competency. Unlike $CaCl_2$ chemically competent cells, TSS cells only need to be resuspended once. I typically expect transformation efficiencies of 10^6 to 10^7 with minimal effort.

Materials and Reagents For a 50 mL outgrowth, yields about 50 aliquots. Scale as necessary

- 5 mL of overnight culture
- 50 mL LB media
- 5 mL 10X TSS buffer
- 250 mL Eulemeyer flask
- at least one 50 mL conical tube
- 0.6 mL Eppendorf tubes
- Incubator shaker
- Ice bucket with Ice
- Refrigerated conical tube centrifuge
- Dry ice bath or liquid nitrogen

Protocol

1. Inoculate 1% v/v of overnight culture in to 250 mL flask
2. Chill centrifuge to 4°C
3. Grow cells to OD:600 0.35 - 0.45
4. Transfer to 50 mL conical tube and chill outgrowth on ice for about 5 min
5. Spin down cells in chilled centrifuge, 5000 rcf for 10 min
6. Prepare dry ice bath/liquid nitrogen bath

7. Decant/pippette away supernatant
8. Gently resuspend cell pellet in 5 mL 10X TSS
9. Aliquot 100 uL of resuspension into 0.6 mL eppendorf tube and snap freeze
10. Store cells at -80°C

5.3.4 Transformation with TSS chemically competent cell

Materials and Reagents For 1 transformation, scale as necessary

- TSS chemically competent cell aliquot
- 5X KCM
- dI H_2O
- Transformation tube - Ideally 15mL polystyrene tube
- LB media
- 42°C water bath
- Ice bucket with Ice
- Incubator shaker

KCM competent cell transformation protocol - Assumes 1-10 uL of sample

1. Let TSS chemically competent cell aliquot thaw on ice
2. Aliquot 20uL of 5X KCM into transformation tube

   Note: Adding competent cells directly to KCM may lower transformation efficiency.
3. Add 80 - (sample volume) uL of dI H_2O
4. Incubate on ice for 0-30 min
5. Heatshock at 42°C fro 40-45s

6. Incubate on ice for 0.5-2 min
7. Add 300uL LB
8. Outgrow cells for 0-1hr
9. Plate cells

5.3.5 Preparation of electrically competent cells

Materials and Reagents - 60 mL of outgrowth yields about 1-2 aliquots of electrically competent cells.

- A lot of sterile 10% glycerol, scales to outgrowth volume.
- Overnight culture at least 1% v/v of outgrowth volume
- Flask large enough to accommodate outgrowth volume
- One 15 mL conical tube
- 50 - large volume centrifuge tubes - scale as necessary given outgrowth volume
- 0.2 mL Eppendorf tubes
- Incubator shaker
- Ice bucket with Ice
- Refrigerated conical tube centrifuge
- Dry ice bath or liquid nitrogen

Preparation of electrically competent cell protocol

1. Inoculate 1% v/v overnight vulture into outgrowth flask with media
2. Chill centrifuge to 4°C
3. Chill sterile 10% glycerol solution on ice

4. Culture cells to OD 0.35 - 0.45
5. Chill on ice for about 5-10 min
6. spin down cells at 5000 rcf for 10 min
7. Decant supernatant, pellet should be stiff so easily pour off supernatant.
8. Resuspend pellet in 10% glycerol, fill container with sterile 10% glycerol
9. spin down cells at 5000 rcf for 10 min
10. Carefully decant supernatant, pellet will be looser.
11. Resuspend pellet in 10% glycerol, fill container with sterile 10% glycerol
12. spin down cells at 5000 rcf for 10 min
13. Carefully decant supernatant, pellet will be looser.
14. Resuspend pellet in about 5 mL 10% glycerol, Pellet should readily dissipate with addition of glycerol.
15. If pellet does not resuspend easily, repeat 10% glycerol wash step
16. Otherwise, transfer 5 mL resuspension to a 15 mL conical tube.
17. Fill 15 ml conical tube with 10% glycerol
18. Spin down at 5000 rcf for 10 min
19. Pippet off supernatant
20. Resuspend pellet in very small volume of 10% glycerol
21. Inoculate 1 uL of resuspension into 1 mL of LB and measure OD:600.
22. Assay for an OD:600 of about 0.15. If too high (0.2-0.3 for standard preparation), add more 10% glycerol. If too low, fill 15 mL conical tube with 10% glycerol and spin down for 5000 rcf for 10 min, remove supernatant, and try again.
23. Aliquote 50 uL of final resuspension and snap freeze
24. Store at -80°C

5.3.6 Preparation of a 96-well plate assay

A 96 well bulk induction assay is a good strategy for assaying a gradient of experimental conditions.

5.3.7 Default Phusion DNA Polymerase Protocol

Adapted with minor modifications from default PCR protocol in NEB phusion manual. Annealing temperature and DMSO may need to be adjusted for optimal results.

1. 98°C for 5:00
2. 98°C for 5:00
3. 55°C for 0:45
4. 72°C for 15s/kb, 45s min
5. Go to step 2 20 times

   Note: Can scale cycle count to for desired yield, 20 is usually more than enough.

6. 72°C for 7 min
7. 18°C forever

5.3.8 Default Taq DNA polymerase Protocol

Adapted from PCR Protocol with Taq DNA polymerase for colony PCR

1. 95°C for 5:00
2. 95°C for 5:00

3. 55°C for 0:45
4. 68°C for 1min/kb
5. Go to step 2 30 times

   Note: 30 cycles for CPCR, 20 cycles is sufficient for amplification

6. 68°C for 7 min
7. 18°C forever