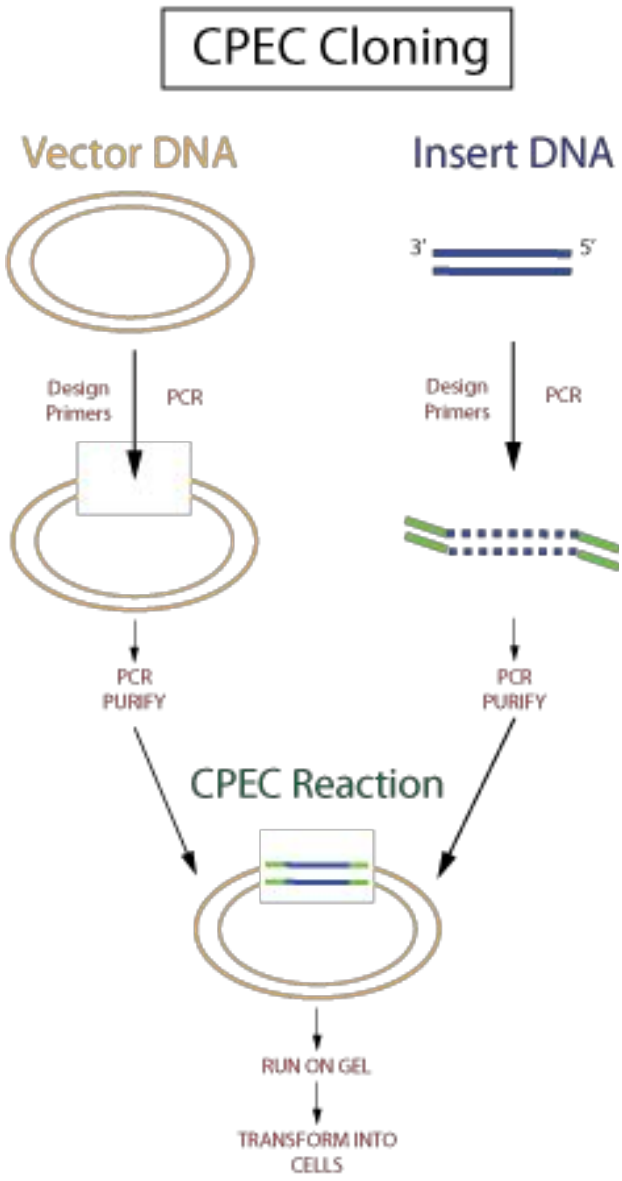
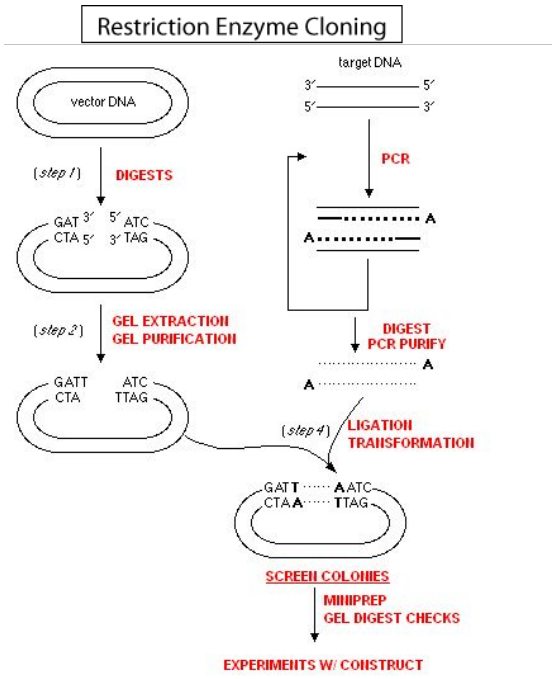
Lab Protocols

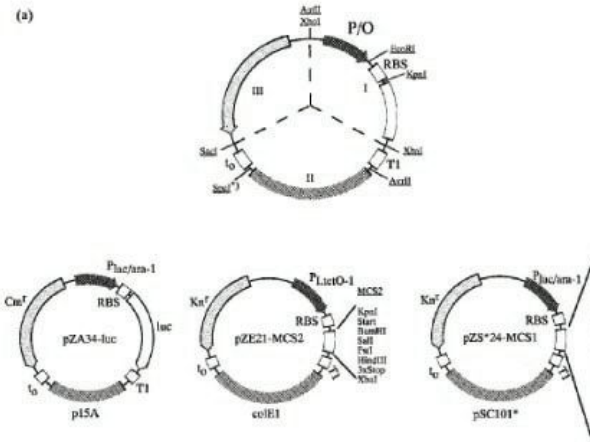
Overview of Molecular Cloning





pZ Plasmid System
Nucleic Acids Res. 1997 Mar 15;25(6):1203-10.

(a)



) Not present in plasmids containing a pSC101 or pSC101 origin

(b)

origin of replication		resistance marker		regulatory unit		designation of vector
E	ColE1	1	Ampicillin	1	$P_{LtetO-1}$	pZE11-
A	p15A	2	Kanamycin	2	$P_{LlacO-1}$	pZA22-
S	pSC101	3	Chloramphenicol	3	$P_{A1lacO-1}$	pZS33-
S*	pSC101*	4	Spectinomycin	4	$P_{lac/ara-1}$	pZS*44-
		5	Zeocin	5	pluxI (w/luxR)	pZS*35sspB
				6	pN25	pN25tetR

Expanded Naming Set of pZ plasmids (Tal Danino, 4/27/2011)

Sm = pSC101m (Jesse's higher copy variation , 10-15 copies)(note this looks more like pSC101 than pSC101*)
5a = Lux/Tet Hybrid Promoter
7=Parsenic promoter
8=PL promoter (lambda)

QIAprep Miniprep

[Designed for the purification of up to 20 µg high-copy plasmid DNA from 1-5 ml overnight *E.coli* culture in LB medium]

14mL culture tube protocol: (Convenient when doing many minipreps)

1. Spin down culture tubes in big centrifuge for 5 mins. [14mL culture tubes can be spun down in big centrifuge up to 3g]
2. Pour supernatant (&tip) into bleach flask, being careful to remove all liquid. If liquid remains in tube then use pipette to remove excess.
3. Add 250 uL buffer P1 to each tube.
4. Place tubes on rack and vortex entire rack slowly till resuspended (watch that fluid doesn't reach top of tube, usually setting 4-6)
5. Transfer 300uL to 1.5 mL microcentrifuge tube.

This replaces steps 1-4 below.

Original Protocol:

· **NOTE**: Use Blue spin columns; centrifuge rpm 14000

1. Pipette 1.5ml from the test tube used for growth and transfer to clean 1.5ml microcentrifuge tube.
2. Spin down cells for 9-10 seconds @ 14000 rpm and remove LB medium.
3. Add 1.5 mL of culture again & repeat.
4. Re-suspend pelleted bacterial cells (via vortexing) in 200 µl Buffer P1 (located in right-most fridge)
5. Add 250 µl Buffer P2 and mix gently by inverting tube 4-6 times. Solution should turn blue.
6. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. Solution should turn colorless.
7. Centrifuge for 10 min.
8. Pipette (700ul) the supernatant into the QIAprep spin column.
9. Centrifuge for 60 sec. Discard the flow-through.
10. Wash QIAprep by adding 0.70 ml (700 µl) Buffer PE and centrifuge 60 sec.
11. Discard the flow-through, and centrifuge for an additional 2 min to remove residual wash buffer. Wait 2-3 mins to dry (removes excess PE buffer and gives better sequencing results).
12. To elute DNA, place QIAprep column in a clean microcentrifuge tube. Add 30 µl Buffer EB to center of each spin column, let stand for 1 min, and centrifuge for 1 min.
13. NanoDrop to find concentration.

High volume minipreps: If plasmid is low copy (i.e. SC101) or a high amount/concentration is

desired, grow 50 mL of cell cultures and use this modified miniprep protocol: I typically only do 10mL of culture per tube to allow for clean minipreps. In the past I've had trouble sequencing preps with too much culture volume, or I see very high bands of DNA on my gels (probably genomic DNA).

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For 50mL's culture
A) Spin down in big centrifuge for 5 mins, remove media thoroughly
B) Add 7.5x amount of P1 (1875 uL)
C) Resuspend, and aliquot to 5 tubes (400 uL each)
D) Add 1.5x amount of P2 (375uL), invert gently 4-6 times
E) Add 1.5x amount of P3 (525uL), invert throughly
F) Spin Down for 10 mins
G) Run supernatant through 3-5 columns
H) Elute with 50-100uL

Notes: Also, after step (F), I sometimes collect the supernatant of microcentrifuge tubes into a big tube to prevent contamination with cell debris. Sometimes I do PE buffer wash twice when a high culture volume is used as well.

NanoDrop Spectrophotometer

Designed to find the concentration (in ng/µl) of DNA.

Procedure:

- **NOTE**: Use 2 µl of liquid for each measurement

1. Blank the instrument using Buffer EB.
2. Drop liquid onto meter and press “measure.”
3. Write concentration on side of microcentrifuge tube – “[concentration]”.
4. Wipe liquid off of bottom- and top-half of meter using Kimwipe.
5. Repeat steps 2-4 for each solution.

Agarose Gel Electrophoresis

Designed to separate DNA, RNA, or protein molecules using an electric current applied to a gel matrix.

"Creating the Gel" Procedure:

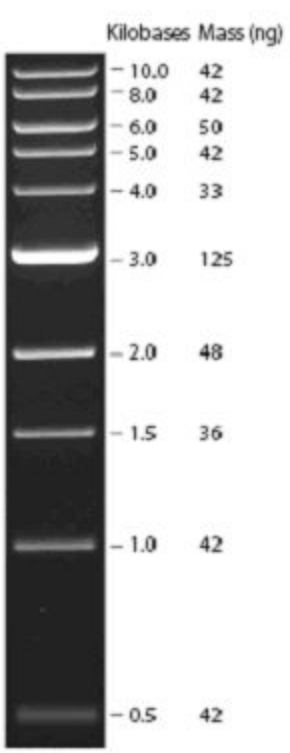
- **CAUTION**: Ethidium Bromide (EtBr) is a carcinogen. When handling, be sure to wear gloves and clean any spills thoroughly.

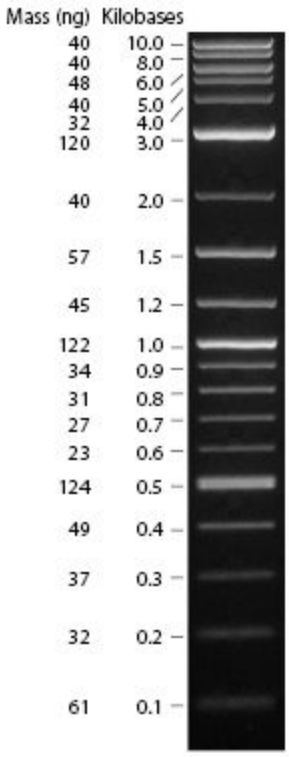
1. Weigh 0.35 g of agarose powder and pour into "EtBr" flask (For a standard 0.7% w/v gel)
2. Measure 50 ml of Buffer TAE and pour into "EtBr" flask.
3. Heat "EtBr" flask in lab microwave for 1 min 15 sec, or until all agarose particles dissolve.
4. Remove flask from microwave using "hot hands" and swirl (careful for vigorous bubbling).
5. Take plastic gel tray and ensure it is tight on gel caster. Insert desired gel comb into grooves.
6. Pipette 5 µl of GelRed dye into flask and swirl to ensure uniformity.
7. Pour contents of flask into plastic gel tray and let stand for ~30 min to solidify.

"Running the Gel" Procedure:

- **NOTE**: Loading dye is "6x," meaning if there is 5 µl of DNA to be tested, 1 µl of dye should be added. Ladder used is 1kb (1000 base pairs). Both dye and ladder are located in third-to-right fridge.
- **NOTE**: Gel may be run @ 110 volts for 40 min(gel extractions, more careful gels) or @ 150 volts for 20 min (diagnostics/quick checks)

1. Remove tape and well placers from gel tray.
2. Place gel tray into electrophoresis apparatus, ensuring that Buffer TAE covers the gel entirely.
3. Mix liquid DNA solution and dye. Solution should turn blue/violet.
4. Pipette liquid (generally 10-15 µl) into respective wells. Begin and end with ladder.
5. Place cover onto electrophoresis apparatus, matching red to red, black to black, and press "run."





1kb Ladder ---------- 2 Log ladder

"Assessing the Results" Procedure:

· **CAUTION**: Be sure to turn off UV lamp when done with photo-capture machine. If gel is to be saved for extraction, limit gel exposure to UV light.

· **NOTE**: Gel will be prone to slide off tray when wet.

1. Remove gel tray from electrophoresis apparatus, letting buffer drip off.
2. Wipe bottom of gel tray and place in photo-capture machine (located near lab microwave).
3. Focus and adjust light (white) of photo.
4. Turn on UV lamp and capture photo.
5. Press save and dump gel into designated bucket located to right of machine.
6. Access and print the photo through server online at ftp://toggle.ucsd.edu. Label wells and compare results with the ladder in order to designate size of DNA fragments.

Special Considerations:

For high separation of small fragments in the range of 0-1000bp, use a 1% gel. For separation of large fragments use a 0.5% gel.

Gel Digests

To cut DNA at specific sequences and often to leave sticky ends for ligating pieces together.

There are two types of Digests we do with restriction enzymes:
(1) screening/diagnostic of colonies
(2) digesting PCRs/plasmids for gel extractions&ligations.

(1) For diagnostic gels, we use between 50-200ng of DNA and digest for 1/2-1 hour before running on a gel. This is just to check if our DNA has the correct fragments
(2) For gel extractions, we use between 1500-3000ng of DNA and digest for 2-3 hours before running on a gel. The gel extraction procedure has low yield thus a lot is needed to start with.

Type (1) Diagnostic Gels

- MIX: Typically 1.0-4.0 microliters DNA, 1.0 of each 10x buffer (check chart), 0.25-0.5 of each enzyme, fill up to 10.0 microliters total with water.
- Leave at 37 incubator for 1/2-1 hour
- Make gel with thin comb
- By the time gel solidifies, probably ready to run gel

Typical Mix is
2.0 DNA
1.0 Buf 2
1.0 BSA
0.5 KpnI
5.5 H20
10.0 TOTAL

Type (2) Gel Extractions

- Digest desired amount of DNA (will probably be around 30-60 microliters) for 2-3 hours at 37.
- Make wide gel comb
- Run gel till bands are well separated and cut out gel piece, trying to minimize amount of agarose

Typical Mix
46.0 DNA
6.0 Buf 2
6.0 BSA
1.0 KpnI
1.0 MluI
60.0 TOTAL

Note: After creating mix, vortex briefly, then spin down briefly to ensure consistency.
Note: Addition of BSA does not affect digests so if 1 enzyme requires it, just add it.

Creating Master Mixes

Often times a large number of samples are digested with the same enzymes & buffers, etc., so

a mix is created for all of them, then aliquoted into the tubes. This is to ensure uniformity across samples and allow for ease of pipetting. For instance if your gel digest mix is:

2.5 DNA
1.0 Buf 2
0.25 KpnI
6.25 H20

and you need this for 9 different samples of DNA, then your master mix is created for 10 samples without the DNA in it (always +1 or +2 the # of samples) :

10.0 Buf 2
2.5 KpnI
62.5 H20
=75 / 10=7.5 microliters/reaction

Before using, vortex & mini-centrifuge your mix to get the liquid to the bottom & ensure it is well mixed.

Keep on ice until aliquoted.
The same technique is done for PCR mixes.

Gel Purification

Designed to purify nucleic acids from an excised gel fragment

1. Weigh excised DNA (TARE with blank tube)
2. Add 3 gel volumes buffer QG(i.e. if piece is 100mg, add 300uL QG).
3. Allow to dissolve at 50 degrees for ~10 mins, vortexing every few mins
4. If gel piece is <500bp or >4000bp, add 1 gel volume of isopropanol & mix
5. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 sec. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 0.7 ml (700 µl) Buffer PE to the column and centrifuge for 60 sec. Discard flow-through and place the column back into the same tube.
7. Centrifuge for the column for an additional for 2 min.
8. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube
9. Wait ~5 min to dry.
10. To elute DNA, add 30 µl Buffer EB (water if no buffer) to the center of the QIAquick membrane, let stand 1 min, and then centrifuge 1 min.
11. NanoDrop to find concentration.

Ligations

To ligate fragments of DNA together.
Typically do a 10.0 microliter mix, with 30-100ng of vector. It is important to do the ligation at a 3:1 or 6:1 insert: vector ratio.

Ligation mix:

- 1.0 T4 ligase buf (make sure thawed, and no flakes at bottom of tube)
- 0.5 T4 ligase
- 2.5-8.5 microliters of DNA pieces at 3:1 molar ratio
- fill up to 10.0 microliters of water

Allow reaction to go on bench top for 30mins-3 hours. Alternatively, leave reaction in 16C water bath for >2-3 hours or overnight.

Calculating vector ratio example:
vector = 15ng / uL, 3 kb size
insert =30 ng/ uL, 1 kb

vector molarity=15/3=5
insert molarity=30/1=30

Here we can do 6:1 insert: vector and add equal amounts of vector & insert. It is probably easiest to add 4.25 microliters of both vector and insert to the ligation mix.

Calculation
For numbers that don't quite work out as nicely, it may be helpful to use the following formula:

$$vol_{vector} = \frac{8.5}{1+\frac{3 \cdot [vector] \cdot bp_{insert}}{[insert] \cdot bp_{vector}}}$$

and

$$vol_{insert} = 8.5 - vol_{vector}$$

where the 8.5 can be switched out for a lower volume (if vector concentration is high), and the 3 can be switched for a 6 for a 6:1 ratio, as opposed to a 3:1 ratio.

Transformations

The genetic alteration of a cell resulting from the uptake, genomic incorporation, and expression of foreign DNA.

- Competence: the ability of a cell to take up extracellular DNA from its environment.
- **NOTE**: Two kinds of cells employed: Bought ($15/each, stronger comp.) and Made-in-Lab ($0.15/each, weaker comp.). Located in -80 C freezer, Made Mach1/DH5alpha cells are in "DH5alphaZ1" box, unlabelled tubes with 70-100 µl of cells in each tube. Bought Mach1 cells are in red Invitrogen box, unlabelled tubes with 25 µl of cells in each tube.e

Procedure:

1. Thaw cells on ice for ~5 min. Ice located down the hall near -80C freezer.
2. Pipette ligated DNA into cell tubes. Add no more than 10% of total volume (e.g. if cell volume is 50 µl, add no more than 5 µl ligated DNA). Swirl solution with tip, DO NOT pipette up and down as it will damage cells.
3. Leave on ice for 30 min.
4. Heat shock @ 42 C for 90sec (30 sec for supercomps).
5. Leave on ice for 2 min.
6. Add 500 µl SOC.
7. Grow for 1 hour by placing tubes within flask and incubating inside shifting 37 C° incubator.
8. While growing, heat appropriate resistance growth plates in 37 C° incubator.
9. When hour is done, spin down cells @ 14000 rpm for 9-10 sec.
10. Remove 400 µl of SOC and re-suspend cells (via vortexing).
11. Plate ~75 µl of cells.
12. Leave rest of cells on bench (in case plated on wrong resistance, can replate in the morning)
13. Put plate in 37C incubator O/N.

PCR protocol

[To amplify fragments of DNA]

<u>New PCR Mix:</u> This mix is a little better and allows you to keep primers undiluted and gives more product for CPEC's

Phusion: Keep in -20 holder, add to mix last

1. PCR mix (amounts in microlters.
    a. 20.0 HF buffer
    b. 2.0 dNTPS
    c. 0.5 Phusion (polymerase)
    d. 0.5 primer –S (undiluted, 100uM)
    e. 0.5 primer –AS (undiluted, 100uM)
    f. ~1.0-2.0 template DNA (need 20-40ng of DNA)
        i) If template is at X concentration, make a dilution to get it to 20-40ng/uL.
    g. 75.0 qH20

<u>Old PCR Mix:</u> PCR primers should be at concentration 10.0 uM

Before Making Mix Below: Prepare primers

We buy primers at 100.0 uM (pmol/microliter) concentration. For the mix below they need to be at 10.0 uM (10x dilution).

- Label a new 0.6 mL microcentrifuge tube with the primer name.
- Add 90 microliters buffer EB
- Add 10.0 microliters of primer
- Vortex briefly

For Phusion: Keep in -20 holder, add to mix last

1. PCR mix (amounts in microlters.
    a. 10.0 HF buffer
    b. 1.0 dNTPS
    c. 0.5 Phusion (polymerase)
    d. 2.5 primer –S
    e. 2.5 primer –AS
    f. 0.5-1.0 template DNA (need 10-20ng of DNA)
        i) If template is at X concentration, make a dilution to get it to 10-20ng/uL.
    g. 33.0 qH20

Common protocol

98ºC 2mins, [initialization]

32x
98ºC 10 sec [denaturation]
50-65ºC 15-30 sec [annealing]
72ºC for 15-20sec/kb [extension]

72ºC for 40sec/kb.

-Typically we do 2 reactions at Ta=56, 60 for our primers designed at Tm of 55. For primers designed at 57 , I typically find Ta=58 & 62 are good Ta's to use. This varies depending on the reaction.
-For plasmid PCR's, I find 15sec/kb is good, for genomic PCRs, I will typically use 20-30secs/kb.

-Annealing time of 20sec is what I start with standardly, but if lower/higher specificity is needed you can change this.

PCR Digests
Before using PCR products in a ligation, products must be digested. If PCR template is same resistance as final construct, digest with DpnI with the additional enzymes as well. DpnI digests methylated template DNA and reduces background.

CPEC Reactions

Cloning method for assembling PCR fragments together. I typically set my overlapping regions to a Tm of ~60-65C and my PCR annealing region to a Tm of ~57-60C.

OLD: PCR purify each piece , then setup a PCR reaction with these requirements:
NEW: Gel Purify each piece , then setup a PCR reaction with these requirements:

- about ~300ng of vector
- about ~200ng of insert
- 1:1 molar ratio of pieces
- PCR mix (amounts in microliters)
    a. 10.0 HF buffer
    b. 1.0 dNTPS
    c. 0.5 Phusion (polymerase)
    d. PCR fragments
    g. fill up to 50 with qH20

CPEC protocol is:
98C 30sect

30 cycles of:
98C 10sec
55C 30sec
72C 20sec/kb

72C 5mins

Before running CPEC protocol, save 10 uL on ice(freezer) as a control.
After CPEC is done, run 10 microliters of the CPEC reaction with the control in the next lane. Check that you can see your assembled reaction on the gel, or that your inserts are less visible than the control (they assembled). If so, then transform 10 microliters into regular competent cells.

Note: In some situations it is difficult to fulfill the first 2 requirements (200 ng insert & 300 ng vector). In those cases I would just make sure there is enough vector, and add enough insert at a insert:vector of (1:1 - 3:1)

Note2: I've found recently that Gel Purifying the vectors gives much higher efficiency and much cleaner results when running the CPEC on a gel. This seems to reduces smears in some CPEC reactions or strange sized pieces(also what they do in paper). I would recommend running PCRs of 50-100 microliters and then gel extracting directly after the PCR instead of gel checking & PCR purifying.

PCR Purification

Designed to quickly purify nucleic acids.

Procedure:

- **NOTE**: Use Pink spin columns; centrifuge rpm 14000.

1. Add 5 volumes of Buffer PBI to 1 volume of the PCR reaction and mix.
2. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 sec. Discard flow-through and place QIAquick column back into the same tube.
3. To wash, add 0.7 ml (700 µl) Buffer PE to the column and centrifuge for 60 sec. Discard flow-through and place the column back into the same tube.
4. Centrifuge for the column for an additional for 2 min.
5. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube. Wait ~3-5 min to dry.
6. To elute DNA, add 30 µl Buffer EB (water if no buffer) to the center of the QIAquick membrane, let stand 1 min, and then centrifuge 1 min.
7. NanoDrop to find concentration.

Sequencing DNA (Eton Bio's service)

To sequence a piece of the plasmid DNA starting from a primer binding site. Sequence generated is typically 1000 bp if successful, 400-500 bp if mildly successful. I typically design my sequencing primers with Tm=55-60C and about 50-100bp upstream of desired sequence. Results should arrive next day.

Protocol

-Aliquot 250-500ng of DNA to be sequenced in a new microcentrifuge tube and label
[NOTE: to qualify for free repeats, aliquot at least 600 ng DNA in 6 uL ddH_2O per reaction, for a concentration of 100ng/uL]
-Aliquot 1 microliter of 100uM primer to a new microcentrifugetube, add 19 microliters of qH20, and label (only need to give them 3-4 microliters of this mix)
-Put both tubes in -20 freezer in the "Eton" box
-Fill out online order form http://etonbio.com/plslogin.php (U/N: hastysequence@gmail.com, pw: pfbh402)
--Check box for primer & DNA template separate
--Fill out concentrations of DNA and primer (primer concentration should now be 5pmol/microliter)
--Fill in your email

Colony PCR

To screen many colonies by PCR'ing a part of the inserted piece

PCR Mix
0.2 dNTPs
0.2 Taq
2 PCR 10x buffer
0.2 -S primer (1o um conc)
0.2 -AS primer
17 water
---
20 total

1) Aliquot mixture to PCR strips
2) Touch colony with Red pipette tip, then let tip stand in tube for ~1 minute
2a) For higher copy plasmids, you might want to dilute into 20 microliters water, and add 1 microliter as template
3) Create master plate with tip for each colony , place at 37 C
4) Run standard PCR protocol but (98 30sec, 50 30sec, 72 1min/kb)x30
5) Run 15 microliters on gel

Alternatively use Taq Master Mix:

8.5 microliters mix
0.2 -S primer (10 um conc)
0.2 -AS primer
1 cell colony diluted by 20-100x
---
10 total

Hotstar Taq MasterMix:
5 uL mix
0.2 -S primer
0.2 -AS primer
1.1 ul 30x diluted colony
4 uL H2O
---
10 total

PCR optimization

Below is a guide for troubleshooting PCR reactions in various situations. My standard PCRs are with the mixes described above.

Scenario: No product
Troubleshooting steps (in order of importance, but many can be done at same time):
-Check that primers sequences bind sequence file at correct location. Check primer Tm's are <3C apart.
-Redo PCR(sanity check). Use original sources, check DNA template concentration(should use 10-20ng/50uL).
-Do a gradient of temps from the Tm (or a few degrees below) to about 5 degrees above.
-Increase annealing time to 30 sec, & do gradient of temps
-Increase/Decrease template concentration by factor of 2-3 (up to 50ng), important for longer PCRs where molarity changes. Or increase/decrease primer concentrations.
-If you have a control plasmid that primers should bind use this as a test
-Use a test primer for the -S & -AS and see which primer isn't working (or order a new one)
-Optimize Mg2+ concentration. 1) Decrease Phusion to 0.25 uL. or 2) Increase Mg2+ in 0.2mM steps
-Sequence template to determine if primer site is there.
-Try DMSO addition to 0-5%

Scenario: Unspecific bands (longer than desired product)
-Check for primer binding sequences that are similar in Vector NTI
-If band is longer than desired product, shorten PCR extension till band goes away
-Increase annealing temperature, search for optimal Ta
-Shorten annealing time
-Use touchdown PCR technique (Start at Ta~+10C of your initial Ta, then decrease by 1-2C/cycle on machine, do 35x cycles of PCR)

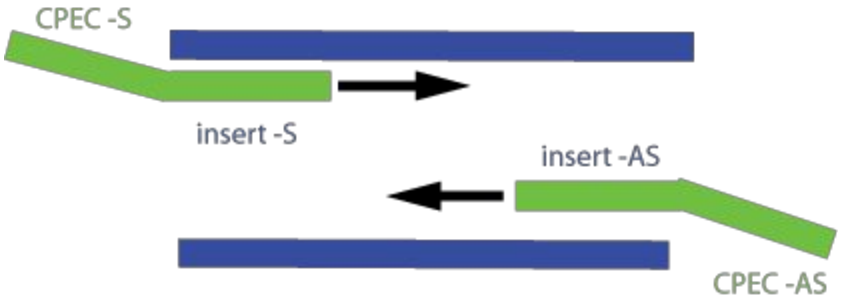
Scenario: Unspecific bands (shorter than desired product)
-Check for primer binding sequences that are similar in Vector NTI
-Increase annealing temperature, search for optimal Ta
-Shorten annealing time
-Use touchdown PCR technique (Start at Ta~+10C of your initial Ta, then decrease by 1-2C/cycle on machine, do 35x cycles of PCR)

Scenario: Primer dimers
-optimize as in "No product" section
-I typically find for this situation Mg2+ concentration & primer/template ratio are most important factors
Scenario: Smears
-typically poor reaction conditions (get new stocks of buffers, dNTPs, etc.)

A good reference from NEB: http://www.neb.com/nebecomm/products/protocol222.asp
Phusion manual: http://www.neb.com/nebecomm/ManualFiles/manualF-553.pdf

Designing PCR Primers for CPEC

For my CPEC reactions I design CPEC ends on the inserts only and PCR the vector w/o CPEC ends. The pZ vectors that are PCR'd can then be added to any insert interchangeably.



I typically follow 3 requirements for designing primers:
(1) Primer Tm about 60C
(2) Primer pair < 3C apart
(3) GC content 40-60% (roughly, though can be stretched)
(4) CPEC ends Tm 60-65C

Step 1: Build desired sequence in Vector NTI
Step 2: Build insert -S primer
(a1) copy first ~25 bp of insert sequence into Finnzymes Tm calculator (link) - should start with ATG/GTG
(b1) remove bases from the **end** of the sequence till Tm is about ~60C
(c1) save part of sequence
Build CPEC -S overhang
(a2) copy 25 bp of sequence upstream of insert
(b2) remove bases from **front** of sequence till Tm is about ~60-65C
(c2) save sequence
Combine sequences in order of (c2) then (c1) for complete -S primer

Step 3: Build insert -AS primer
(a3) copy last ~25 bp of insert sequence into Finnzymes Tm caclculator (link) - should end with TAA/TAG/TGA
(b3) remove bases from the **front** of the sequence till Tm is about ~60C
(c3) reverse complement sequence (link)
(d3)save reverse complemented sequence
Build CPEC -AS overhang
(a4) copy 25 bp of sequence directly after insert
(b4) remove bases from **end** of sequence till Tm is about ~60-65C
(c4) reverse complement sequence (link)
(d4) save reverse complemented sequence
Combine sequences in order of (d4) then (d3) for complete -AS primer

Step 4: Error checking

(1) make sure insert binding sequences binds to separate insert plasmid source within a few mismatches

(2) make sure full primer sequences bind assembled Vector NTI sequence

Put sequences into ordering site (set Normalization YES, 100uM):
Valugene: https://valuegene.com/process.cgi

pZ plasmid Construction Notes

For simple gene insertion into pZ plasmid with original RBS , here are the CPEC ends:
CPEC -S: attaaagaggagaaaggtacc (add to beginning of insert -S primer)

for LAA tagged inserts:
CPEC -AS: TCGTCGTTCGCTGC (add to beginning of insert -AS primer)

for non-LAA tagged inserts:
CPEC -AS: gcctctagcacgcgt (add to beginning of insert -AS primer)

Vector Primers

LAA vector primers: ZE24EcoRBS orig vect -AS , pZE25vect -S
noLAA vector primers: ZE24EcoRBS orig vect -AS , pZE25vectnoLAA -S

Combining pZ plasmids

I use the following set of primers with a synthetic linker following the AvrII site which seems to combine these plasmids efficiently. I have similar primers for various origins & promoters as well:

Pa/I vect -S tgtatagtcacgactggtcg cctagggcgttcgg
Pa/I vect -AS CCAGTTGATCGACGATTC tctagggcggcgg
pLtet cfp -s GAATCGTCGATCAACTGG CTAAGAAACCATTATTATCATGACA
pLtet cfp -as cgaccagtcgtgactataca gTCTAGGGCGGCG

Screening colonies from CPEC Reactions

After miniprepping several colonies from your CPEC reaction, choosing the proper restriction enzymes to distinguish between correct and incorrect colonies is crucial. The most important considerations are:

1. What are the possible incorrect plasmids? Where did insert & vector pieces come from and if it is possible that they were transformed (i.e., they have the same resistance, etc.) ?
2. How big are the fragments being generated with restriction enzymes used? Will it help distinguish between incorrect/correct plasmids? Keep in mind larger fragments are harder to differentiate.
3. Will the fragments generated be visible on the gel? Keep in mind the lower limit of detection is 25-50ng of DNA with GelRed.

Below is an example screening for a typical CPEC reaction. Here is the chart I usually setup before doing a CPEC:

8.24.2010	**PCR Name**	**PRIMERS**	**TEMPLATE**	**SIZE**	**RESISTANCE**
CPEC S: pZA11sfGFP					**Amp**
S1	**pZA11vect**	**ze24 EcoRBSorig vect -AS, pZE25 vect -S**	**pZA11yfp**	**2.2**	**Amp**
S2	**sfGFP**	**RBSorig sfGFP -S, sfGFP LAA/AAV -AS**	**pZE24sGFP-LAA**	**0.7**	**Kan**

Consideration 1: Determining incorrect / correct plasmids

The final correct plasmid here (pZA11sfGFP) is Amp resistant. Hypothetically, the templates from fragments S1 & S2 can be transformed at a low level, but in this case we see that piece S2 (template pZE24sfGFP-LAA) cannot be transformed because it is Kan resistant. Thus the only possible incorrect template that could be transformed is pZA11yfp. In addition, CPEC reactions often times give a background of closed vector, i.e., the S1 PCR closes on itself and is transformed. Since this is Amp resistant as well this can be a possible incorrect plasmid (call it pZA11empty).

This gives us three possible cases of plasmids that could be transformed:

1. Correct plasmid -- pZA11sfGFP
2. Background plasmid from S1 -- pZA11yfp
3. Background closed vector -- pZA11empty

We need to choose a restriction enzyme(s) that will help us distinguish between these cases. The best way to do this is to use or construct sequences for each of the three cases and then write down the size of the fragments from Vector NTI.

Constructing Sequences
Use primer sequences from above to search for where primers bind on the initial template sequence of vector & insert, S1 & S2(only use binding part not CPEC overhangs). For the vector, delete the rest of the bases and save the sequence (this will be your pZA11empty sequence). Then use Copy & Paste commands to put the insert sequence into your vector and save as final plasmid (pZA11tetR). You should now have the three sequence files to digest with.

Common Digest types for screening
Single cut: If we choose an enzyme which cuts once on each of these vectors we can often screen this way. In this situation, the sizes of the fragments would be (1) 3.01, (2) 3.02 (3)2.4 kb. We can differentiate here between (1)&(2) vs. (3) but not between (1) & (2) on a gel, thus this is not an ideal screen for this situation.

Insert cut: One of the simplest options is usually to try and cut out the insert (sfGFP, yfp, or empty) and check the corresponding size of the fragment. Here sfGFP and YFP both have sizes of 750bp, thus it is not a good option for this situation.

Interior cut(s): Another way would be to find an enzyme cutting inside of either tetR or yfp, and screen this way.
I typically don't like to see a situation where 1 of the plasmids is uncut because this does not control against the reaction failing altogether. However, it may be possible to find an enzyme which cuts once in yfp/sfGFP, and twice in the other, allowing us to distinguish the two situations. Looking at both sequences in Vector NTI, and adding common "Restriction Sites" we can see that there are two XhoI cuts in sfGFP while only one in the YFP plasmid. This can be used as a screen between the two cases.

Restriction Summary of Multiple Plasmids: We can use this site and input the plasmids to highlight the differences in the restriction sites between 2 or 3 plasmids. Enter them in the form:
>Plasmid 1
atatatggg
>Plasmid 2
atatatttt
>Plasmid 3

Consideration 2: Distinguishing fragments
Digests of the 3 plasmids with XhoI gives the following fragments

1. 500bp , 2500bp
2. 3000bp
3. 2200bp

These can be distinguished on a 0.7% gel pretty easily.

Consideration 3: Visualizing fragments
Typically we do something between 100-200 ng of DNA for gel digest checks. To be more precise, what we mean is that we need each fragment cut out to be > 50 ng to be visualized on a gel, and usually 100-200 ng total DNA covers this requirement. Since smaller pieces of DNA are harder to visualize (less mass & less dye bound), we need to be careful about being able to visualize the 500 bp piece. In addition, since our screen is based mostly on being able to see the 500bp or not, it is important to make sure we get this right.

If we want the 500 bp piece to be approximately 50 ng's, and it is about 1/6 of the total mass of the plasmid, then for this digest it would be safe to start with about ~300 ng's of starting DNA. If DNA is concentration of minipreps is in the range of 40-50 ng/uL for instance, we could do something like 6.5-7.5 uL of DNA for each. This will also make the master mix simpler since no water will be added.

Notes:
Need to find a website which can do a Restriction Summary on multiple plasmids at once, highlighting difference in cuts.

Making Chemically Competent Cells

0. Start an O/N culture of your strain in 1-5mL LB media.

1. 1/100x dilution of overnight culture in 25 mL LB (makes 25 competent cells). Grow up for ~2-3 hours(depending on strain), OD needs to be between 0.4-0.6 for significant competency.
-Immediately put on ice once it's reached above OD.
2. Spin down cells at 8000 for 5 min. Resuspend in 2.5 mL TSS solution(1/10x original). Aliquot to prechilled microcentrifuge tubes (prechill at -20C). Freeze at -80C for storage (retain competency for about ~6 months)

Making PDMS chips for Microscope Experiments

Tal Danino

Overall, during this procedure you want to be as CLEAN and CAREFUL as possible with making chips.
-Wafers are very fragile and should not be bent. Best to hold them on sides away from features, and kept in a clean petri dish when not in use.
-During mixing & the rest of chip making you want to be careful about getting dust in your mix or on your chips, and if your gloves get dirty you will want to change them.

Making foil dish for wafer: Put on clean gloves. Cut out about a 8 inch by 8inch piece of foil. Take non-useful wafer and trace the circular shape onto the foil. After this draw a 2 inch circular margin around and cut out with scissors. Flatten and straighten the foil so that there are fewer creases in it.

Making PDMS mix: Take a plastic hexagonal dish (not the top one in the bag), and blow dry with airgun. Tare scale w/dish and 30g of Silicone, then 3 g of curing agent. Clean a glass rod with a kimwipe and clean with blow gun. Under the fume hood, stir mixture for 5 minutes. The goal is to get the mixture as uniform as possible.

Removing bubbles from PDMS mix:
NEW: Pour entire mixture into 50mL Falcon tube. Spin down for 3-5 minutes to remove bubbles.
OLD:Place tray w/ mix in the dessicator on the RIGHT and put on lid. Turn on vacuum pump in the hood while valves are all closed. Make sure back valve(air release) is closed and SLOWLY open up front valve. The lid of the dessicator should be sticking to the blue part, and you will see the gauge go to about ~25 or so. Allow air bubbles to come to surface, and every 2-3 minutes OPEN back valve for a few seconds to release pressure. Do this about 5-10 times or until you see no bubbles.

Pouring PDMS mixture over wafer: Place your desired wafer in the foil dish you made and raise up the sides around it so that you can pour in the PDMS mix. Its best to try and have the wafer lie FLAT on the foil so that PDMS doesnt get in under it. Place tray w/ wafer in the dessicator on the RIGHT. Very slowly pour over the PDMS mix w/o bubbles onto the wafer. Best to pour at center of the wafer and allow it to spread to the sides. REPEAT procedure to remove bubbles from the wafer. There should already be much less so you can leave it going for 5-15 minutes and come back and check if bubbles are gone.

Baking PDMS chips: To harden the chips, bake in the 80 degree oven for 1 hour. Carry over your foil tray w/ wafer & mix to the oven very carefully and try not to introduce more bubbles.

Removing PDMS layer from the chip: After baking, leave dish at room temperature for about ~5minutes. This part you have to be the most careful with since there is a chance of breaking the mask in half or damaging the surface. SLOWLY peel off foil from the PDMS. If you can, try and remove some of the PDMS from the bottom of the wafer at the same time. First you'll want to remove all PDMS from bottom of the wafer. To do this, take a razor blade and slowly cut off the thin layer up until the outside of the circle. Make sure all of the PDMS from the bottom is

removed before trying to lift off the PDMS from the top. VERY VERY SLOWLY lift up the PDMS layer from one side of the chip, and try and do this evenly from all sides. When you peel off the PDMS from the feature part of the chip do this really carefully and allow PDMS to peel off by itself w/o applying a lot of pressure. Make sure that you do not bend the wafer at all, because this can cause it to snap in half. When separated place wafer into a clean dish and set aside.

Punching holes in chips: Cut out excess PDMS and save a smaller square around the chips you want to use so that its easier to work with. Take YELLOW puncher, glass dish w/ rubber bottoms, and tweezers to light scope. Focus on the circular holes and place the puncher directly above the circular part. Make sure you are holding it as vertical as possible, and then press down hard to punch through. Slowly lift up puncher(easier if you turn it back and forth), and remove PMDS from puncher with tweezer. Continue doing this for ALL holes on the chips.

Cleaning holes: (use syringe filled with water to clean out excess PDMS from holes.)
Use the razor to cut out each chip individually. Place chip in clean Petri dish. Plug in port with syringe and apply pressure till water leaves from other side. Do this for all holes and on both sides of the chip.

Cleaning chips: This part is very important so that no dust enters in chips. Place chips in the glass dish (wash w/ water and blow first) and add 70% Ethanol to submerge them. Swirl the chips around in the dish w/ the lid and pour off the excess ethanol. Repeat this rinse with water from milliQ system. After water rinse, individually blow dry each chip on both sides and put in a new Petri dish. For each chip use scotch tape to remove dust from the chip. **Important: Run fingernail over features of the chip several times to remove dust(this seems to be most revelant step for clean chips)**. Leave chip sealed with a fresh piece of scotch tape on the feature side.

Cleaning coverslips:
Be gentle/careful with coverslips, they can break easily and are very sharp. Take a new coverslip and spray on both sides with Heptane. Wipe clean on both sides with a Kimwipe and make sure there are no liquid spots or residues on both sides. Do the same for Methanol. Now wash with water on both sides and then air dry with blow gun. Absolutely make sure there are no spots or dust on both sides (if there are redo water wash), and place clean in a Petri dish.

Bonding chips to coverslips:
Open O2 valve of plasma bonder, and make sure O2 level is between 0.4 to 0.6. Turn on plasma bonder and run for 5 minutes to warm up. Place 1 chip(make SURE feature side is facing up!) and 1 coverslip in bonder tray (use tweezers for coverslips). Run for 3 minutes. When done, open up tray and flip over chip onto coverslip to bond. You'll want to do this as quick as possible for best seal. Place in 80 degree oven overnight in a glass tray (Not Plastic!!)

Microscope Experiment Protocol DAW6v2

Tal Danino 8.26.2009

1. Grow O/N culture from a -80 stock or plate.

2. **Grow up cells.** 2 hours before setting up experiment, do a 1/1000x dilution of cells in appropriate inducers/antibiotics in 50mL media. Grow cells for ~2 hours till OD=0.05-1.0 (I shoot for 0.08), and have chip wetted and setup before spinning down cells.

3. **Prep syringes** (During 2 hours) (2 water, 2media, 2cells). Make sure to use media & waters filtered + 0.075% Tween20. Add dye to one of the media's (1uL per 5mL).

4. **Inspect chips** (During 2 hours) Go to microscope and check out chips at 4x magnification(PhL condenser setting), don't need to screw down chips. Look around for pieces of dust blocking channels that could be a problem. And check to make sure traps are not collapsed (they look collapsed if color of posts look same as traps). Tape the additional device on the chip to prevent from getting contaminated or wetted. Set the temperature of the scope box at this point to with fan at max speed. Screw tight all 4 points on the coverslip holder with red rubber slips evenly.

5. **Wet the chip** from 1 of the 2 media ports and raise the reservoir to the highest position to speed up wetting. Once a port has become wet (looks like no fluid movement on punched hole, or droplet on surface), then plug that port in and raise it up to the highest as well (at same point as other one is ideal). I prefer wetting the chip with the media with dye in it.

6. **Tape lines.** Once all ports are wet, tape down each of the lines to the square microscope insert. Be very gentle when touch lines because bonding of chip can get ruined.

7. Set ports to the appropriate heights. Medias should be a DAW height of 500-550. Shunt (water) at 21-22", Junction (water) at 8".

8. **Spin down cells** for 4 mins in big centrifuge. Dump excess media back into 50mL flask as backup cells and put in 37 shaker. Add 3-5 fresh media and resuspend by vortexing briefly. Prepare syringe as before and set up at proper height (7") and plug in. Set both cell ports at identical heights.

9. Slowly raise the cell ports or lower the junction about 1-2". Watch the cell ports at 20x mag (Ph1 condenser). After approximately ~2-5 mins you'll see cells start to come down from ports and towards the waste. Check the junction and make sure cells are going in there and Mcherry dye is present. Also make sure cell reservoirs are not mixing and both going directly to the junction.

10. **Load cells** Set a slow speed for cells going by the traps and proceed to flicking. The goal is to get at least 1-2 traps loaded for each lane (4 total), and best if they are loaded at traps closest to junction (cause they will fill all the ones downstream). Before flicking, tape down lines. Hold lines taut, and give a few hard flicks, then checking to see what got loaded.

11. If cells are loaded then reverse the flow by raising the heights to previous values. Cells should be seen zooming by traps at a rate of 25-200 um/sec.

12. **Setup DAW.** Go to DAW setup and adjust heights so that its at 50% level. Jog+ one of the heights till it's at the 100% level. This should be extremely close the boundary, but not allow the other media to flow through. Set the 100 % level on software. Do the same for 0 level. And hit Calibrate on DAW software. Test 0, 50, and 100% levels with slider. Create and load DAW run table file.

13. Allow cells to grow inside trap for 3-4 doublings(1-2 hours). Make sure you give them the appropriate media before starting the experiment. In our case, we want to give them 0% Arabinose so first step will induce them.

14. Setup 100x objective. CAREFULLY remove stage insert and put a big drop of oil on 100x objective(Ph3 condenser). Make sure it snugly inserted. Slowly bring up objective till oil touches coverslip. Bring condenser down to level which produces highest amount of lights exposure and hit Auto Exposure.

15. **Set up Scope software**. Select your XY points, time frequency for camera acquisition, and set brightfield / GFP / Mcherry wavelengths. For DAW6v2, shoot for 30-60 seconds for a round of brightfield and fluorescent images (about 3-5 xy points) and set GFP every 6 to 12 frames. Set up autofocus and make sure global settings match for Advanced Brightfield Phase only and not GFP. Use red box for autofocusing over cells. Make sure fluorescent lamp is off & set GFP exposure to desired (1-3seconds). Test a single loop for autofocus to see how long it takes and that it catches the right focus. If focal planes are far away in Z direction adjust allen wrench tightness on one side or carefully push down on stage insert to even setup. Mcherry exposure setting should be set to 200-300 ms and every 12 mins.

16. **Make sure fluorescent lamp is set to 10%. Start the DAW and scope run simultaneously.**

Flow Cytometry

Settings are what I use for typical tagged and untagged FP's in E.coli

1. Turn flow cytometer ON, **then** turn ON Computer (Make sure computer is off first , and I usually wait 30 seconds to turn on the computer)
2. Login: Bridget, Leave PW blank
3. **Setup Software:** Apple-> CellQuest
4. Acquire->Connect to Cytometer
5. File->Open->Istanbul->FACS Calibur Users->Hasty->Tal->"Acquisition Template"
6. Cytometer -> Threshold: FSC-H -> 0; Cytometer ->Detector/Amps -> FL1 Log 750; FSC Log E00; SSC Log 350; Compensation all to 0
7. Acquire->Parameter Description, Hit Folder and make new folder with date. Select.
8. Acquire->Counters
9. Hit RUN on flow cytometer.
10. Acquire while in Setup mode (checked) and adjust flow (LOW/MED/HIGH) so that events are not more than 5000events/sec
11. To acquire data, uncheck Setup, and just go through samples.
12. **Shutdown:** Put in a tube with bleach (follow instructions on paper printout above cytometer)
13. **Transfer Files:** Apple->Recent Servers->Images3. Password is normal complex one.
14. Make new folder in your directory. Copy files over from Istanbul(On desktop)->FACS Calibur Users->Hasty->Tal->Directory. Copying directories doesn't work as well

Preparing Electrocompetent cells and Electroporations

T.D adapted from
http://openwetware.org/wiki/Recombineering/Lambda_red-mediated_gene_replacement

Makes ~7-8 aliquots of e- comp cells.

- Grow 5mL strain of interest with pKD46 at 30C overnight.
- Prepare two flasks with 1/100x dilution of overnight in 250 mL LB and grow at 30C.
- Label 1 flask + and the other - L-arabinose (control)
- When OD_{600} of cells(+pKD46) reaches 0.1 (~1-1.5 hours for JS006-24min doubling), add L-arabinose to concentration of 0.15-1.5% to induce pKD46 λ-red expression
    - add ~2mL of 25% L-arabinose to 250 mL + culture, none to - culture
    - Note: original Datsenko/Wanner protocol did 0.15%, but Moreno2006 reports higher transformants at 1.5%
- Continue to grow at 30°C to OD_{600} = 0.4 (~1-2 hours)
- Chill cells in ice-water bath 10 minutes
- Centrifuge 10 min at 4000rcf 4°C in 35mL nalgene centrifuge tubes (or use sterile 50mL tubes, max spin 4g)
- Pipette off supernatant and resuspend pellets in 1-5 mL ice-cold dH_2O(filtered)
- Centrifuge 10 min at 4000rcf 4°C
- Pipette off all dH20 carefully
- (Optional: another spin wash step in ice-cold dH20)
- Resuspend pellet in 1000 µL dH_20 +10-15% glycerol
- Aliquot 50uL per tube (prechilled)

- **Electroporation**
- For electroporation step, include 2 conditions: +/- PCR fragment
- Chill electroporation cuvettes for 5 minutes on ice(or don't need if kept in -20C)
- Add 5 pg to 0.5 µg PCR amplified DNA to cells (For genomic insertions: Typically I add 50-100ng (of 50-100ng/uL))
    - Moreno2006 reports 500ng works better
- Set electroporation apparatus to "Bacteria"
- Prepare 1mL SOC in pipette. Take cuvette off ice, wipe metal electrodes with kimwipe.
- Place the cuvette into the sample chamber(sort of quickly so no condensation on electrodes happens)
- Apply the pulse by pushing the button
- Remove the cuvette. Immediately add 1 mL ~~LB~~ SOC medium and transfer to a sterile culture tube
- Incubate 60-120 min with moderate shaking at 37°C
- Plate at 37C (for genomic insertions)
- If transformation doesn't work, replate in the morning (as in Datsenko)

General Electroporation

1) Do 1/100x dilution of cells in LB or LB + 1/2 AB's. I usually do 100mL LB to make 6-8 cells.
2) Grow up to very nearly OD600=0.6 (+- 0.05)- Around 2.5-3.5 hours
3) Keep on ice , Spin down in refrigerated centrifuge 5mins
4) Wash with ice cold sterile water 2-3 x
5) Concentrate in 250x ice cold water + 15% glycerol
6) Add 0.5-1.5uL DNA. Electroporate in 0.1mm cuvette, 1.8kV, 200ohms,20uF (bacteria setting)
7) Add 1mL SOC within 30 sec of electroporating (I prepare pipette with media ahead of pulsing it)
8) Time constant check should be 2.5-5ms
9) Grow 1 hour, then plate

For 430 I will get about 10^4-10^5 cells for a 1 plasmid electroporation (50-150ng). 10-100 for 2 plasmid. Rarely any for 3 plasmid. It always helps to use fresh e- comp cells, but if you are just doing 1 plasmid sometimes stored ones are okay. Also best to keep everything cold if you can. I forget exactly how long Step (2) takes but it's around 2.5-4 hours over here.