Sands et al. 2018
Detailed Protocol

Necessary Reagents

LB liquid

LB agar plates with 50ug/ml kanamycin

YPD liquid

YPD agar plates with 200ug/ml G418

Yeast Freezing Media (5%glycerol, 10%DMSO). Make fresh

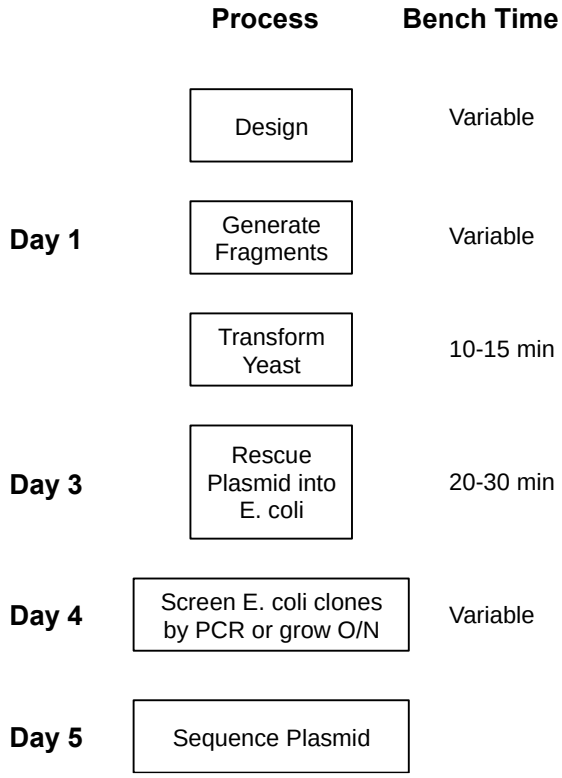
Salmon Sperm DNA (Rockland #MB-103-0025)

Optional Reagents

Sorbitol Buffer (1M Sorbitol, 100mM EDTA)

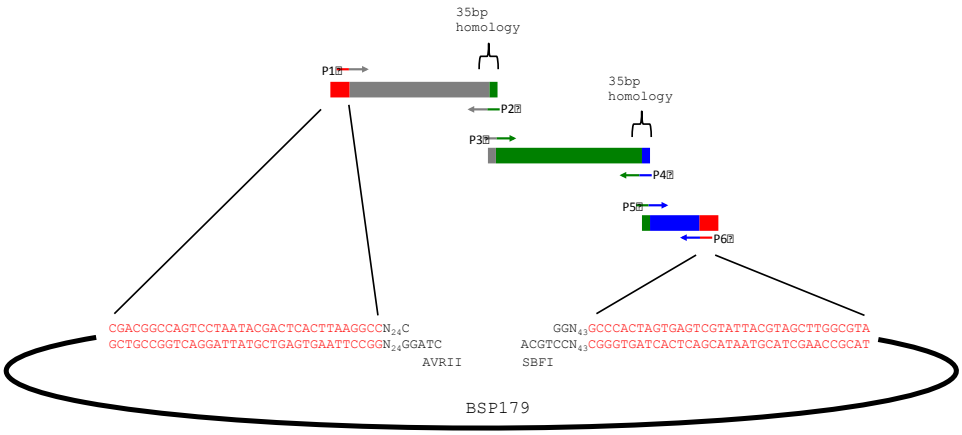
lyticase/Zymolase- Zymoresearch Inc, cat # E-1004

Workflow



Primer Design and Homology Arms

The homology arms at the ends of each fragment will determine how fragments assemble and how all fragments get inserted into the plasmid backbone. There is flexibility here. If you want to use these vectors as MosSCI insertion constructs, you can use any sequences within the MCS as homology arms so long as they are 20-35 bp at each end. If you want to make a complex repair template for CRISPR/CAS9 editing, you would design homology arms outside the MosSCI insertion sequences. The example below is an illustration of how we make MosSCI repair constructs from BSP179.



In this example, BSP179 is digested with AvrII and SbfI leaving overhangs. The homology arms for insertion into the vector are shown in red and are located 24 nucleotides upstream from the AvrII cut site and 43 nucleotides downstream of the SbfI cut site. All intervening nucleotides will be deleted after recombination, so it doesn't matter how long the overhangs are.

This construction requires 6 primers (P1-P6). Therefore, the sequences for them would be the following:

P1 CGACGGCCAGTCCGTAATACGACTCACTTAAGGCCINSERT1SEQUENCE
P2INSERT2HOMOLOGYINSERT1SEQUENCE
P3INSERT1HOMOLOGYINSERT2SEQUENCE
P4INSERT3HOMOLOGYINSERT2SEQUENCE
P5INSERT2HOMOLOGYINSERT3SEQUENCE
P6TACGCCAAGCTACGTAATACGACTCACTAGTGGGCINSERT3SEQUENCE

Preparation of linearized vectors for DNA assembly

Following this protocol gives us near background free vector preps. We use enzymes from New England Biolabs and set up reactions in a theromocycler. Any

enzyme or enzyme combinations within the MCS can be used to linearize the vectors, but we find AvrII and SbfI give us the best results. Digests are set up as follows:

Vector DNA	1.5-3ug
Cutsmart Buffer	5ul
AvrII	2ul
SbfI HF	1ul
dH2O	to 50ul

Incubate for 3 hours at 37^{o} then heat kill the reaction at 80^{o} for 20 minutes.

Run reactions on a 1% agarose gel and extract the linear DNA from the gel.

Purify DNA using the QiaexII kit (Qiagen Inc).

You should recover enough linearized vector for 10 or more DNA assemblies from a single digest.

Note about linearizing constructed plasmids for swapping sequences (ie, dropping a new XFP into your newly constructed plasmid)

In order to linearize constructed plasmids for promoter swaps, XFP swaps, etc., one needs only to linearize the vector within the region to replaced. However, if the new insert has significant sequence homology to the sequence to be replaced, it is better to digest the vector with multiple enzymes that remove as much of the homology as possible. An example of this is when we replace a GFP-derived variant with another GFP-derived variant, say swapping Cerulean for Citrine. Also of note is that many worm reporter genes contain multiple copies of a synthetic intron sequence. These introns sequences are nearly identical in DNA sequence. When we want to swap these reporters for another that also contain many synthetic introns, we find it is important to remove them by restriction digest when making vector preparations. Most of our reporter genes contain just a single intron as we find this to be sufficient for the increased expression associated with introns in transgenes.

Preparation of Competent Yeast

We use frozen, single-use yeast aliquots for all of our DNA assembly reactions. Our protocol is base on (Gietz and Schiestl, 2007). Many different yeast strains can be used for DNA assembly (Gibson, 2011) and in our hands we have not seen a difference in efficiency between haploid and diploid strains. Here, we used BY4741 (Brachmann et al., 1998).

To prepare yeast, inoculate a single colony of Saccharomyces cerevisiae into 5ml of YPD and grow the culture overnight at 30° while rotating. The next day, inoculated 1ml of the overnight culture into 49ml of fresh YPD in a 250ml baffled Erlenmeyer flask and grow at 30° with shaking until the OD600 reaches 1.5-2.5 (usually between 4 and 6 hours).

Harvest cells and pellet in a centrifuge at 4000rpm for 5 minutes. Remove supernatant and wash the pellet in 25ml of sterile dH2O. Spin again as before and remove supernatant. Carefully resuspend the pellet in 370ul of Freeze Media (recipe below) and mix by pipetting up and down slowly trying not to generate bubbles. Once mixed to a single cell suspension, the total volume should be close to 500ul. If needed, add a little more Freeze Media and mix by pipetting up and down. Pipet 50ul of the cell suspension into sterile Eppendorf tubes, place the tube into a "controlled-rate" freezing box, and place at -80. We use a homemade Styrofoam box but a commercial controlled-rate chamber will surely work.

Freeze Media Recipe (make fresh right before freezing)

30% Glycerol	1ml
DMSO	0.6ml
dH2O	4.4ml
Total Volume	6.0ml

DNA Assembly Reactions

Salmon sperm DNA is from Rockland Inc., cat #MB-103-0025. To make single use aliquots of ss DNA for transformation, thaw manufacturers tube and aliquot 60ul (enough for 2 reactions) into PCR tubes. Heat to 98° for 5 minutes then <u>immediately</u> place on ice. Do not allow the tubes to cool slowly in the thermocycler or on bench top. Store at -20°.

Thaw 1 Eppendorf tube of competent yeast per assembly reaction. Thaw tube by holding in hand for 2-3 minutes. Spin in centrifuge at 10,000rpm for 30 seconds to pellet cells. Remove supernatant and then add transformation mix in the following order:

PEG 4000	240ul
10xLiAc	36ul
Salmon sperm DNA	25ul
60-100ng linearized vector	
*insert DNAs	
dH2O	to 360ul total volume

* inserts should be at least 20:1 insert:vector molar ratio.

Vortex on high for 1 minute or until the pellet has been resuspended to single cell suspension.

Place tubes in 42^{o} water bath and incubate for 40 minutes.

Remove tubes from water bath and pipet the full contents of each into culture tubes filled with 3ml YPD.

Incubate tubes for 2-3 hours at 30^{o} while shaking or rotating.

During outgrow, pre-warm selective agar plates (YPD + 200ug/ml G418) to 30^{o}. After outgrow, plate 100-300ul of culture onto each 10cm selective agar plate. Incubate at 30^{o} for 48 hours or until colonies appear.

Generally, we move straight to *E. coli* plasmid rescue at this stage. However, if you want to screen yeast by colony PCR you can do so. Best results are obtained when screening yeast clones from fresh plates (ie, 48 hours after plating and not stored at 4^{o}).

Plasmid Rescue

For plasmid rescue we modified the Qiagen miniprep protocol to include a glass bead disruption step. If glass beads are not available, one can use lyticase to enzymatically digest the yeast cell wall.

Scrape yeast off plates with a pipet tip into 500ul dH20. Try to scrape enough that when spun down you have a 50-150ul equivalent of packed cell pellet.

Spin cells at 10,000rpm for 30 seconds and remove supernatant.

Add 320ul of Buffer P1.

Fill each tube with enough glass beads such that the Buffer P1 meniscus reaches the 500ul line.

Vortex on high for 10 minutes. We use a Vortex Genie (Scientific Industries, Inc).

After vortexing, add 320ul of Buffer P2 and invert the tube 5-10 times to mix. Incubate at room temperature for 5 minutes.

Add 450ul Buffer N3 and mix by inverting the tubes.

Spin tubes at 13,000rpm for 10 minutes.

After centrifugation, carefully pipet liquid phase onto Qiagen miniprep columns. Do not pour the liquid phase onto columns as you will clog the column with glass beads.

Continue as per manufactures protocol for a standard miniprep.

Elute DNA with 30-40ul Buffer EB pre-warmed to 55-60^{o}.

Eluted DNA can be transformed into any *E. coli* strain.

Optional Protocol:

If you do not have access to glass bead disruption or you want to maximize recovery of plasmid DNA (ie., you are working with plasmids over 20Kb), you can digest the yeast cell wall with lyticase (Zymolase) and then continue the rescue miniprep with yeast spheroplasts. To do so, resuspend the yeast pellet in 500ul of Sorbitol buffer and 5ul of Zymolase. Mix by vortexing and then incubate for 30 minutes to 1hr at 30^{o} without shaking. After incubation, spin down spheroplasts for 1 min at 8000rpm. Discard supernatant then continue the rescue as above by adding 320ul of Buffer P1. Skip glass bead step.

References

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14:115-132.

Gibson, D.G. 2011. Gene and genome construction in yeast. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]* Chapter 3:Unit3 22.

Gietz, R.D. and Schiestl, R.H. 2007. Frozen competent yeast cells that can be transformed with high efficiency using the LiAc/SS carrier DNA/PEG method. *Nature protocols* 2:1-4.