

2022 MIT iGEM Protocols

Credits

The *E. coli* cloning protocols in Part 1 are originally from the MIT iGEM 2021 lab protocols, with minor modifications.

The majority of the yeast protocols in Part 2 are taken from Ilya's yeast manual, also with minor modifications.

The MoClo YTK protocols are based on the protocols outlined in [A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly](#).

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Part 1: Cloning

Preparing gBlocks (1 hr)

1. Before opening the tube, centrifuge for 3-5 seconds to ensure the DNA is in the bottom of the tube.
2. Add molecular grade water, or a buffer such as IDTE, to reach a final concentration of 25ng/uL.
 - a. 250 ng: 10 μ L
 - b. 500 ng: 20 μ L
 - c. 1000 ng: 40 μ L
3. Incubate at $\sim 50^{\circ}\text{C}$ for 15-20 min to ensure the solvent comes in contact with the pellet and the entire sample is resuspended. (If having difficulty, try 55°C for 1-5 min.)
4. Store at -20°C .

Marillonnet MoClo Kit pL0 Plasmid Golden Gate Assembly (up to 4 hr)

1. Assemble reaction in the following order:
 - a. 7.5 uL sterile water
 - b. 50 ng of L0 insert (2uL)
 - c. 100 ng of L0 backbone (2uL, use DNA from Deepak and not our minipreps)
 - d. 1.5 uL T4 Ligase buffer
 - e. 1 uL of T4 high-concentration ligase
 - f. 1 uL of BbsI-HF
2. Run following thermocycler program:
 - a. 15 min at 37°C (initial digestion)
 - b. 25 cycles of:
 - i. 2 min at 37°C (digestion)
 - ii. 5 min at 16°C (ligation)
 - c. 15 min at 37°C (digestion)
 - d. 5 min at 50°C (digestion)
 - e. 5 min at 80°C (inactivation)
3. Store at 4°C.

Marillonnet MoClo Kit pL1 and pL2 Plasmid Golden Gate Assembly (4 hr)

1. Assemble reaction in the following order:
 - a. 50 ng of L1 insert (1uL) each
 - b. 50 ng of L1 backbone
 - c. 6.5 uL (variable depending on number of L1 inserts) sterile water
 - d. 1.5 uL T4 Ligase buffer
 - e. 1 uL of BsaI-HF (pL1) or BbsI-HF (pL2)
 - f. 1 uL of T4 high-concentration ligase
2. Run following thermocycler program:
 - a. 15 min at 37°C (initial digestion)
 - b. 25 cycles of:
 - i. 2 min at 37°C (digestion)
 - ii. 5 min at 16°C (ligation)
 - c. 15 min at 37°C (digestion)
 - d. 5 min at 50°C (digestion)
 - e. 5 min at 80°C (inactivation)
3. Store at 4°C.

Transformation (2 hr + 16 hr)

1. Thaw a 25 uL tube of NEB 5-alpha or 10-beta competent cells **for each reaction** on ice for 10 minutes.
 - a. Use 10-beta cells for larger plasmids (>10kb), or plasmids with more repeats. Otherwise, 5-alpha cells should suffice.
2. Warm LB+antibiotic agar plates at room temperature.
3. Add 2 uL of each assembly reaction plasmid and pUC19 to separate cells and gently mix by flicking the tube 4-5 times.
4. Incubate on ice for 30 minutes.
5. Heat shock at 42°C for 30 seconds (exactly).
6. Place the cells back on ice for 5 minutes.
7. Add 950 uL of room temperature **growth media**. Incubate at 37°C for 60 minutes, shaking vigorously (280 rpm).
 - a. Use **Stable Outgrowth Media** if using NEB 10-beta or NEB Stable Cells.
 - b. Use **Super Optimal broth with Catabolite repression (SOC)** if using NEB 5-alpha cells.
8. Mix cells thoroughly by flicking the tube and inverting, then spread 50 uL using glass beads onto LB+antibiotic agar plate.
 - a. Spread a larger amount if transformation efficiency is expected to be low (e.g. many inserts, large plasmid).
 - b. Be sure to use the correct antibiotic; the following is based on the Marillonet MoClo toolkit:
 - i. Spec for pL0
 - ii. Amp for pL1
 - iii. Cam for pL2
9. Incubate the plate overnight at 37 C. Keep the competent cell tube in +4C overnight.
10. Let the plate grow no more than 16 hours. Parafilm and place in a 4°C fridge
 - a. The cold will help set blue color for blue/white screening, and red color may also become more apparent.
11. Bleach competent cell contents with 20% bleach in a 1:1 volume ratio and dispose.

Inoculating (i.e. liquid culture) (10 min + 16 hr)

1. Obtain your plates. Obtain two 14 mL falcon conical tubes per plate and label each tube with date, construct number, contents, and colony #.
 - a. Alternatively: Per plate, obtain ONE disposable culture tube and label each tube with date, construct number, contents, and colony #.
2. Add 4 mL of LB media into each 14 mL tube.
3. Add 4 μ L of appropriate antibiotic solution (spec for pL0, amp for pL1, cam for pL2)
 - a. Make sure you pipet the antibiotic solution into the liquid and NOT the side of the tube. Also make sure you do not lower the tip too far below the surface of liquid to avoid contaminating the pipet.
4. The plates should have a good mix of blue and white colonies. Pick two colonies per plate to inoculate.
 - a. Blue = took up plasmid but not the insert. White = took up plasmid with the insert. Cells without the plasmid will not grow on the antibiotic.
5. For each tube + plate: Using a P200, obtain a clean new tip, open the petri dish and dip the pipette tip into the colony.
 - a. It is important to avoid colony cross contamination—more important that getting a lot of bacteria on your pipette tip.
6. Take the tip and lower it into the 4mL culture, being careful not to submerge the entire tip. Discard the tip into the biowaste bin.
7. Gather tubes and grow for no more than 16 hours at 37°C at 280 rpm.

Making glycerol stocks

1. To a cryovial, add equal parts of 40% glycerol and culture. 750 uL of each should be enough.
 - a. Label tubes with date, colony #, and part ID, e.g. pL0[egfp] 08/30 #2
2. Incubate at room temperature for 30 minutes.
3. Transfer to -80°C freezer.
 - a. Recommended to keep an unverified glycerol stock box, and once sequencing results come back toss all incorrect glycerol stocks.

Miniprep

Miniprep: [NEB Monarch Plasmid Miniprep Kit](#) (2 hr)

*All centrifugation steps should be carried out at 16,000 x g (~13,000 rpm).

If precipitate has formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.

Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

1. Pellet 1–5 ml bacterial culture (not to exceed 15 OD units) by centrifugation for 30 seconds. Discard the supernatant.
2. Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1) (pink). Vortex or pipet to ensure cells are completely resuspended. There should be no visible clumps.
3. Lyse cells by adding 200 µl Plasmid Lysis Buffer (B2) (blue/green). Invert the tube immediately and gently 5–6 times until color changes to dark pink and the solution is clear and viscous. Do not vortex! Incubate for one minute.
 - a. Do not incubate for longer than one minute to prevent plasmid denaturation. Do not handle the sample roughly or chromosomal DNA may copurify as a contaminant.
4. Neutralize the lysate by adding 400 µl of Plasmid Neutralization Buffer (B3) (yellow). Gently invert the tube until color is uniformly yellow and a precipitate forms. Do not vortex! Incubate for 2 minutes.
5. Clarify the lysate by spinning for 5 minutes at 16,000 x g.
6. Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.
7. Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Centrifuge for 1 minute. Discard the flow-through.
 - a. The collection tube is designed to hold 800 µl of flow-through fluid and still allow the tip of the column to be safely above the top of the liquid. Empty the tube after each step to ensure no contamination.
8. Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.
9. Label a clean 1.7 mL tube and transfer the column to it.
 - a. Use care to ensure that the tip of the column has not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute before inserting it into the clean microfuge tube.
10. Add 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.
 - a. Delivery of the Monarch DNA Elution Buffer should be made directly to

the center of the column to ensure the matrix is completely covered for maximal efficiency of elution.

11. Use a spectrophotometer to obtain concentration and purity of your DNA

Miniprep: Qiagen (2 hr)

1. Pellet 1 mL bacterial overnight culture by centrifugation at 6800g for 3 minutes. Discard the supernatant.
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. (after making glycerol stocks, it's okay to pellet all remaining culture)
 - a. Ensure that RNase A has been added to Buffer P1.
 - b. No cell clumps should be visible after resuspension of the pellet.
 - c. If LyseBlue reagent has been added to Buffer P1, before use vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved.
 - d. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
3. Add 250 µl Buffer P2 and mix thoroughly by gently inverting the tube 4–6 times. Continue inverting the tube until the solution becomes viscous and slightly clear. **Do not allow the lysis reaction to proceed for more than 5 min.**
 - a. Do not vortex, because this will result in shearing of genomic DNA and contamination of plasmid. Continue inverting the tube until the solution becomes viscous and slightly clear.
 - b. If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2.
 - c. Mixing should result in a homogeneously colored suspension.
 - d. If the suspension contains localized colorless regions, or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
4. Add 350 µl Buffer N3. Mix immediately and thoroughly by inverting the tube 4–6 times. The solution should become cloudy.
 - a. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3.
 - b. Large culture volumes (e.g., ≥5 ml) may require inverting up to 10 times.
 - c. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue is gone and the suspension is colorless.
 - d. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
6. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 Spin Column by pipetting.

- a. Avoid touching the pellet at all costs, even if it means not getting all 800 μ L
7. Centrifuge for 30–60 s. Discard the flow through.
8. Recommended: Wash the QIAprep 2.0 Spin Column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow through.
 - a. This step is necessary to remove trace nuclease activity when using endA+ strains, such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains, such as XL-1 Blue and DH5 α , do not require this additional wash step.
9. Wash QIAprep 2.0 Spin Column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
10. Discard the flow through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.
 - a. Important: Residual wash buffer will not be completely removed unless the flow through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
11. Place the QIAprep 2.0 Spin Column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μ l Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep 2.0 Spin Column, let stand for 1 min, and centrifuge for 1 min.

Preparing LB+antibiotic plates

1. Add 7.25 g LB to 500 mL water. Stir. Add 7.25 g [Bacto Agar](#), stirring.
 - a. Regular agarose (Agarose RA, for example) can also be used,
2. Split into multiple flasks, 1 for each category of antibiotic plate you are making.
3. Autoclave for 15 minutes at 121°C ("Liquid A" on the Tuttnauer LabSci 15+L).
4. Add appropriate antibiotics once the solution is cool enough to touch. Keep stirring or place in a water bath until ready to pour.
5. Make the following 1 mL stock solutions of antibiotic (1000x)
 - a. Kan - 50 mg/mL (50 mg in 1 mL water)
 - b. Spec - 50 mg/mL (50 mg in 1 mL water)
 - c. Amp - 100 mg/mL (100 mg in 1 mL water)
 - d. Cam - 25 mg/mL (25 mg in 1 mL water)
 - e. MIs - 50 mg/mL (50 mg in 1 mL water)
6. Add antibiotics in a 1:1000 ratio to the total volume.
7. Pour the plates.

Preparing stock solutions

LB growth media

1. Suspend 25 g in 1 L of distilled water.
2. Autoclave for 15 minutes at 121 °C.

Preparing 1% agarose electrophoresis gel

Materials

- Agarose
 - suitable options: [VWR Agarose RA](#)
- 1x TAE buffer

Protocol

1. For a 1% agarose gel, mix 0.75 g of agarose with 75 mL of 1x TAE in a microwaveable flask. Add the agarose to the flask before the TAE.
2. Microwave in pulses for 1-3 min until the agarose is completely dissolved
 - a. Do not over-boil the solution since some of the buffer will evaporate and alter the final percentage of agarose in the gel
3. Let agarose cool down to ~50C (about 5 min, when you can comfortably keep your hand on the flask)
4. Add 7.5 uL of 10,000x SYBR safe stain.
 - a. **OR** optionally, add ethidium bromide (mutagen!) to a final concentration of 0.2-0.5 ug/mL to visualize the DNA under UV light
5. Pour the agarose into a gel tray with the well comb in place
 - a. Pour slowly to avoid bubbles!
6. Set for ~45 minutes.

Gel electrophoresis

1. Once gel is set, remove combs upwards carefully. Make sure the casting wells are filled with 1x TAE.
2. Add loading dye to samples (in a ~1:5 or 1:4 ratio).
3. Load gels with samples. **Remember to add a DNA ladder!** Follow manufacturer recommendations (~5-20 uL load).
4. Attach red/black lead, making sure the positive lead is placed opposite from the loading wells.
5. Set gel apparatus to 115V and start the electrophoresis. Check that there are bubbles in the buffer and that wire leads are secured.

Each gel runs for 45 to 60 minutes. Do not let the dye front run off the gel! You can image the gel under UV light after to confirm correct digestion.

Restriction digest

Note: You want ~0.75 to 1.5 µg DNA for every 20 µL digestion reaction.

1. Each PCR tube gets:
 - a. 2 uL 10x NEB CutSmart Buffer
 - b. x uL of DNA plasmid corresponding to ~0.75-1.5 ug DNA
 - c. y uL of sterile H₂O to bring reaction to 20 uL
 - d. 1 uL per enzyme used
2. Add water first, then DNA, then buffer, then enzyme.
3. Place tubes in the 37 °C incubator. If enzymes show a “time saving icon,” incubate for 30 minutes. Otherwise, incubate for 60 minutes.
4. Add 4 uL 6x loading dye to stop the reaction.

Go to <http://nebcloner.neb.com/>.

PCR (using Q5 2x Master Mix)

1. To a PCR tube, add:
 - a. 10 μ L of 1 μ M forward and reverse primer
 - b. 10 μ L of Q5
 - c. 1 μ L of template DNA

Colony PCR

1. To a PCR tube, add:
 - a. 10 uL of 1uM forward and reverse primer
 - b. 10 uL of Q5
2. Using a toothpick or inoculating loop, pick up some of the colony, patch it on a plate and then add to the PCR tube.

PCR cleanup

Note: All centrifugation steps should be carried out at 16,000g.

1. Dilute sample with DNA Cleanup Binding Buffer (ensure that isopropanol has been added, as indicated on the bottle label)* according to the table below. Mix well by pipetting up and down or flicking the tube. Do not vortex. A starting sample volume of 20–100 μ l is recommended.

SAMPLE TYPE	RATIO OF BINDING BUFFER:SAMPLE	EXAMPLE
dsDNA > 2 kb (plasmids, gDNA)	2:1	200 μ l:100 μ l
dsDNA < 2 kb (some amplicons, fragments)	5:1	500 μ l:100 μ l
ssDNA > 200 nt**	7:1	700 μ l:100 μ l

1. Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute, then discard flow-through.
2. Re-insert column into collection tube. Add 200 μ l DNA Wash Buffer and spin for 1 minute. Discarding flow-through is optional.
3. Repeat wash (Step 3).
4. Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 1 minute to ensure traces of salt and ethanol are not carried over to the next step.
5. Add ≥ 6 μ l of DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.

Note: Typical elution volumes are 6–20 μ l. Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield. Care should be used to ensure the elution buffer is delivered onto the matrix and not the wall of the column to maximize elution efficiency.

gDNA (genomic DNA) Extraction: [QIAamp Mini kit](#)


QIAGEN Product ID	51306
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1. Inoculate single colony in 2-3 mL LB. Shake 2-3 hours at 37°C; culture should be cloudy.
2. Spin down 1-1.5 mL in an Eppendorf tube for 2 min at full speed, discard supernatant
 - a. Freeze pellet at -20°C or continue with prep
3. Resuspending pellet in 180 µL lysis buffer with 40 mg/mL lysozyme. Use P1000 since the buffer is super bubbly. Vortex vigorously.
4. Incubate at 37°C for 30 minutes
5. Add 25 µL Proteinase K and 200 µL Buffer AL. Use P1000 for buffer AL (also super bubbly). VORTEX.
6. Incubate at 70°C for 30 min (CAP TIGHTLY)
7. Add 20 µL 100% ethanol. VORTEX.
8. Apply all of the sample to the DNeasy column. Centrifuge for 1 min at 6000g.
9. Transfer column to new collection tube
10. Wash column with 500 µL Buffer AW1. Centrifuge for 1 min at 6000g. Transfer column to a fresh collection tube.
11. Wash column with 500 µL Buffer AW2. Centrifuge for 3 min at max speed. Transfer column to a clean Eppendorf tube for sample elution.
12. Add 200 µL Buffer AE to the column. Wait 1 min at room temp. Centrifuge at 6000g for 1 min to collect DNA.

Additional note on the Mini vs. Micro kits — the Mini kit is used for extracting gDNA from tissue and cells.

Features	QIAamp DNA Micro Kit	QIAamp DNA Mini Kit
Applications	Real-time PCR, STR analysis, LMD-PCR	PCR, Southern blotting
Elution volume	20–100 µl	50–200 µl
Format	Spin column	Spin column
Main sample type	Whole blood	Whole blood, tissue, cells
Processing	Manual (centrifugation or vacuum)	Manual (centrifugation or vacuum)
Purification of total RNA, miRNA, poly A+ mRNA, DNA or protein	Genomic DNA, mitochondrial DNA	Genomic DNA, mitochondrial DNA, bacterial DNA, parasite DNA, viral DNA
Sample amount	1–100 µl	200 µl/25 mg/5 x 10 ⁶
Technology	Silica technology	Silica technology
Time per run or per prep	30 minutes	20 minutes
Yield	<3 µg	4–30 µg

Gibson Assembly with [NEB Gibson Assembly® Master Mix / Gibson Assembly® Cloning Kit](#) (incomplete)

- Designing Gibson primers: <http://nebuilder.neb.com/#!/>
- https://www.neb.com/-/media/nebus/files/manuals/manuale2611_e5510.pdf?rev=c2a9c78a94af4ee29da34b62cf4ddef2&hash=AD2431F95B100290CBFBEEEDB6AB0F807
- Gibson assembly worksheet for determining reaction amounts:
 Gibson Assembly Worksheet

DpnI digestion

1. In a total 10 µl reaction, mix 5–8 µl of PCR product with 1 ul of 10X Cutsmart and 1 ul (20 units) of DpnI.
2. Incubate at 37°C for 30 minutes.
3. Heat-inactivate DpnI by incubating at 80°C for 20 minutes.
4. Proceed with the Gibson Assembly Cloning procedure, described below.

Gibson assembly

1. Thaw chemically competent cells on ice.
2. Add 2 µl of the chilled assembly product to the competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tubes to ice for 2 minutes.
6. Add 950 µl of room-temperature SOC media to the tube.
7. Incubate the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the selection plates. Use Amp plates for NEBuilder Positive Control sample.
10. Incubate overnight at 37°C.

NEB HiFi DNA Assembly

- <https://www.neb.com/protocols/2014/11/26/nebuilder-hifi-dna-assembly-reaction-protocol>

Prepping samples for Plasmidsaurus (incomplete)

- Ensure caps fit snugly and close them firmly
- Wrap caps in a light layer of parafilm to keep the caps closed
- Place the wrapped tubes into a small protective vessel (such as a Falcon tube or small box) to prevent the tubes from opening or being crushed during shipping.
- Include a sheet with your name, number of samples, and order label along with your tubes. Either print out a copy of the order confirmation you receive upon your submission, or write out that information on a piece of paper

(am dumping stuff from lab notebook, will re-organize later)

pJUMP digestion with SpeI ()

Protocol

Want ~0.75-1-1.5 ug DNA for every 20 uL digestion reaction

1. Each PCR tube gets:
 - a. 2 uL 10x NEB CutSmart Buffer
 - b. **X uL pJUMP plasmid DNA (19.652 ng/uL)**
 - c. Sterile H₂O (up to 20 uL total reaction volume)
 - d. 1 uL SpeI enzyme
2. Add water first, then DNA, then buffer, then enzyme
3. Place tubes in 37C incubator: if enzymes show 'time saving icon,' for 30min. Else, 60 min.
 - a. Even if enzyme has "time saving icon", incubate for 60 min
4. Take a **X uL** aliquot of your digest + move it to a separate tube where you will add 5x loading dye in a 1:5 ratio (this sample will later be run on a gel) to inactivate the enzyme
5. Incubate the remaining digest at 80 C for 20 min (heat inactivation for SpeI-HF)
6. Store digest product in -20 C

Notes: should end up with TWO tubes, one will be stored in -20 C, and the other sample will be run on a gel

PCR lacZdropout from pL1f1 ()

For a 50 uL reaction (using [Q5 2X Master Mix](#)):

1. Each PCR tube gets:
 - a. 25 uL Q5 High Fidelity 2X Master Mix
 - b. 2.5 uL 10 uM forward primer
 - c. 2.5 uL 10 uM reverse primer
 - d. **X uL pL1f1 template DNA**
 - i. For a 50 uL reaction, NEB recommends **1 pg - 10 ng** of plasmid template DNA
 - e. Nuclease-free H₂O (up to 50 uL)
2. Transfer PCR tubes to a thermocycler and run with the following conditions (recommended by NEB):
 - a. (98 C, 30 sec), (98 C, 5-10 sec; 72 C, 20 sec; 72 C, 25 sec/kb)x25-35, (72 C, 2 min), hold 4 C

Running pJUMP digestion + lacZdropout PCR product on a gel ()

Follow standard protocol for [pouring a gel](#)

1. If you haven't already, add loading dye to the pJUMP digestion aliquot in a 1:5 ratio
2. Add 5X loading dye in a 1:5 ratio to the lacZ PCR product

- a. Note: you will have to load this sample into multiple lanes since a single well can comfortably hold ~20-25 uL

Follow standard protocol for [running a gel](#)

Construct ID (contents)	Predicted fragment sizes (bps)	Actual size estimates
pUMP digestion		

See virtual digest + gel below:

Gel extraction (lacZdropout PCR product) ()

1. After imaging the gel, while it is still on the UV transilluminator, use a clean razor blade to carefully excise the band corresponding to the expected PCR product size
 - a. Try not to include a lot of excess gel when cutting out the band
2. Place the excised bands in clean, labeled microcentrifuge tubes
3. Follow instructions from **[name of gel extraction kit in BMS]**

Gibson assembly (pJUMP + lacZdropout PCR product) ()

Protocol

Using [HiFi DNA Assembly Mix](#); for a 2-3 fragment assembly:

1. Add the following to one PCR tube:
 - a. 10 uL HiFi DNA Assembly Master Mix
 - b. Sterile H₂O (up to 20 uL total reaction volume)
 - c. X uL pJUMP digest (50-100 ng)
 - d. **X uL** lacZdropout PCR product (2X excess to vector)
2. Incubate sample in a thermocycler at 50 C for 15 minutes
3. Store samples on ice or at -20 C for subsequent transformation

Part 2: Yeast Protocols

“MoClo YTK” refers to the MoClo Yeast Toolkit created by the Dueber Lab: [A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly \(Lee et al., ACS Synth. Biol. 2015\)](#).

MoClo YTK: Assembling pL1 plasmids

Required materials

- 0.5 uL of each part
- 1 uL T4 Ligase buffer
- 0.5 uL T4 DNA ligase
- 0.5 uL **Bsal**
- nuclease free H₂O (up to 10 uL total reaction volume)

Protocol

1. Assemble reaction with following components:
 - a. 0.5 uL of each YTK part
 - b. 1 uL T4 DNA Ligase Buffer
 - c. 0.5 uL T4 DNA Ligase
 - d. 0.5 uL **Bsal**
 - e. nuclease-free H₂O (up to 10 uL total reaction volume)
2. Run the following program on the thermocycler:
 - a. 25 cycles of digestion and ligation (42 °C for 2 min, 16 °C for 5 min).
 - b. **If assembling a cassette such as pYTK096, skip the final digestion and heat inactivation** since GFP dropout will contain Bsal cut sites that should not be cut.
 - i. Otherwise, include a final digestion step (60 °C for 10 min) and a heat inactivation step (80 °C for 10 min).
3. Store at 4°C.

MoClo YTK: Constructing new pL0 parts

Yeast Transformation (16 hr + 4 hr + 16 hr)

Day 1

1. **On the day before transformation**, inoculate your strain into 4 mL liquid culture overnight.
 - CSM selective media if the strain already contains plasmids
 - +GAL if with Cas9 plasmid

Day 2

2. **Next day**, take an OD₆₀₀ measurement and dilute your sample to ~0.1 OD₆₀₀ in 10x mL media in a disposable culture tube or Erlenmeyer flask. Prepare 10mL per transformation.
3. Give at least 2 replication cycles and check the OD₆₀₀: anywhere between 0.4-1.0 OD₆₀₀ is good (ideal: 0.5, late exponential phase).
 - Per 1 replication: 90 min in YPD or 120 min in CSM
 - Total time: 3 to 4 hours
4. After 150 minutes into step 3, turn on 100°C and 42°C baths.
5. Place your salmon sperm in 100°C for 5 min. Place on ice afterwards.
6. Take **10⁸ cells** (= 5 ODs), pellet in the **floor centrifuge at 3000 g** for 5 min.
 - There are 2×10^7 cells in 1 mL of 1.0 OD₆₀₀ culture.
 - It is OK to use fewer cells for plasmid transformations.
7. Aspirate the medium, wash in 10 mL sterile water, and pellet again.
8. Aspirate the supernatant, resuspend the pellet in 1.0 mL sterile water, and transfer to a 1.5 mL tube.
9. Pellet in **tabletop centrifuge at 13,000 g** for 15 s. Aspirate the supernatant.
10. Layer (no mixing):
 - 240 µL 50% PEG
 - Provides a protective layer between cells and toxic LiAc
 - 36 µL 1M Lithium Acetate
 - 10 µL boiled salmon sperm DNA
 - If transforming PCR product: 50 µL PCR product
 - If transforming a plasmid: 1 µL plasmid
 - (optional) 74 µL sterile H₂O
11. Use a 1000 µL pipette to break up the pellet, then vortex to uniformly resuspend.
12. Incubate at 42°C for 40 min.
13. Pellet in **tabletop centrifuge at 13,000 g** for 30 s, remove the supernatant with a micropipette, and resuspend in 200 µL YPD.
14. Plate 200 µL on selective media for auxotrophy, or non-selective (e.g. YPD) media for a drug.

- In drug-containing media, the drug is going to start killing right away before the resistance gene has been expressed \Rightarrow cells are dead.
 - Instead, first plate on regular media, then restreak to drug media.
- On auxotrophies, the cells won't immediately die from lack of just one nutrient — they'll just start off growing slowly and then express the selection marker gene.

15. Incubate plates in 30°C overnight.

Revision history

Date	Details
2022-08-10	- Use a pipette to break up the pellet, <i>then</i> vortex to uniformly resuspend.
2022-08-09	- Modify dilution to detail 10mL per transformation.
2022-08-08	- Give length of time for boiling salmon sperm DNA. - Give specific <i>g</i> forces for centrifugation.

References

- [High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method](#)
- Ilya Andreev

Quick & Dirty Genomic DNA Extraction

Reagents

- 0.2 M Lithium acetate (dilute 1 M sol'n) + 1% SDS solution (powder)
 - (e.g. 1 mL 1 M LiAc + 4 mL H₂O + 0.05g SDS = 5 mL total)
 - 1% = 1 g / 100 mL
- 96-100% ethanol and 70% ethanol solutions.

Protocol

1. Pick one yeast colony from a plate or spin down 100-200 uL of liquid yeast culture (OD ~ 0.4). Resuspend cells in 100 uL of 200 mM LiAc + 1% SDS solution
2. Incubate for 5 min at 70 C in a thermomixer
3. Add 300 uL of 96-100% ethanol
4. Vortex 10 sec
5. Spin down DNA and cell debris at 15,000 g for 3 min
6. Pour supernatant out into liquid waste or aspirate it
7. Wash pellet with 300 uL 70% ethanol
 - Vortex 2 s
8. Centrifuge 5 min at full speed
9. Remove supernatant, being careful not to disturb the pellet
10. Centrifuge again briefly and remove residual liquid with a pipet (20 uL)
11. Let air dry 5-15 min
12. Dissolve pellet in 75 uL of TE
13. Spin down cell debris for 15 s at 15,000 g

Use 1 uL of supernatant for PCR

Gel Electrophoresis

Creating a Gel

- Mix 0.375 g (.75%) Molecular Biology-grade agarose in 50 mL 1xTAE
 - Use 0.75 g (1.5%) agarose for smaller (<500 bp) inserts
- Microwave for 1.5 to 2 min, swirling periodically until all agarose is dissolved
- Wait until the flask is cool enough to touch, then add 2.5 μ L Ethidium Bromide stock (20,000x)
 - EtBr is a known mutagen, so all EtBr materials go into designated waste bucket
 - EtBr is temperature- and UV-sensitive
 - If using SYBR Safe (which is also mutagenic), use 5 μ L.
- Insert desired combs, pour into the gel mold, and wait 15-25 min for gel to solidify.

Running a Gel

- Loading instructions
 - 6 μ L 1kb Ladder
 - (<30) μ L reaction contents + 4 μ L loading dye
 - Dye with SDS produces sharper bands as it helps remove DNA-bound proteins, e.g. restriction enzymes
 - The prominent red/pink band migrates similarly to Bromophenol Blue (approximately 400 bp on a 1% agarose gel). The much lighter blue dye migrates to approximately 4000 bp on an agarose gel
 - Pipet carefully into wells
 - Make extra space between wells if product is extracted later
- Fill the reservoir with 1xTAE buffer to the fill line
 - Filled TAE in the tank can be reused until it becomes yellowish
- Standard runtime: 40 min at 90 V

Gel Extraction

- Carefully lift your gel with the mold out of the reservoir and place it onto a clean wipe
- Grab razors or cover slips, a clean 1.5 mL tube per each sample, and march your way into the Dark Room
- Take a picture of your gel using the gel imager and save it
- Proceed with extraction on the transilluminator
 - Always wear a face shield and protective gloves!
 - Removing as much empty gel as possible around your band will improve yield
- Once samples are extracted, follow the QIAGEN Gel Extraction Kit protocol
 - If your chunks are too big, transfer into 15 mL falcon tubes

- *For improved sample yield (may or may not work):*
 - *Binding step* – lower centrifuge speed (2,000 RPM)
 - *Elution step* – warm EB to 52 C, elute twice (30 + 30 uL), and increase incubation time to 5 min

Preparing 40% glucose solution (500 mL)

Note: The protocol for 40% *galactose* is exactly the same, but with “glucose” replaced with “galactose” below. However, note that some people (you know who you are) believe that 40% galactose solution is physically impossible to make. To be on the safe side, opt for 20% galactose for your experiments.

- Add 250 mL of distilled H₂O water into a 1L beaker, add a stir bar
- Add first 100 g glucose, *slowly*, make sure that *no precipitate* forms at the bottom, especially around the corners (swirl w/ steel spatula) - otherwise you will get rock candy!
- Add the other 100 g, keep stirring, heater can be set to 50-60 C
- Total volume = volume H₂O + **volume glucose**, has to be 500 mL, so transfer to a graduated cylinder and bring it up to 500 mL with water, if needed
- Parafilm the cylinder, invert several times to make sure the mixture is uniform
- Aliquot, preferably 125 mL in 250 mL flasks
- Autoclave 20 min
- If there is undissolved sugar left, swirl the (hot) bottle until all extra sugar is dissolved
 - Especially relevant when making galactose

Preparing 20% galactose solution (500 mL)

Preparing YPD (Rich Media for yeast, 500 mL)

Plates

1. In a 1L conical flask, mix the following:
 - 5 g Yeast Extract
 - 10 g Peptone (tryptone works as well)
 - 10 g agar (Bacto Agar preferred)
 - Stir bar
 - 470 mL Milli-Q water
2. If preparing liquid media, transfer to a sufficiently sized bottle.
3. Autoclave for 15 min (liquids cycle).
4. After autoclaving, add 25 mL 40% glucose solution and place on a stirrer for a slow, gentle stir.
5. Let stand for 20-30 min on a stirrer until flask cool enough to touch, then pour the plates

Liquid media

1. In a 1L conical flask, mix the following:
 - 5 g Yeast Extract
 - 10 g Peptone (tryptone works as well)
 - 470 mL Milli-Q water
 - Stir bar
 - If your lab already has premixed broth such as [Fisher BioReagents™ YPD Broth \(Powder/Molecular Genetics\)](#), simply use the concentration provided by the supplier (e.g. 50 g/L for Fisher YPD Broth).
2. If preparing liquid media, transfer to a sufficiently sized bottle.
3. Autoclave for 15 min (liquids cycle).
4. After autoclaving, add 25 mL 40% glucose solution. If using premixed broth, check to see if glucose/dextrose is already added. If so, skip this step.

Revision history

Date	Details
2022-08-08	<ul style="list-style-type: none">- Change autoclave time from 20 min to 15 min to match Tuttnauer Liquid A.- Split protocol into plates and liquid media.- Add note on premixed broth.

Preparing CSM (“Complete Supplement Mixture”) (500 mL)

Plates

Materials

- CSM mix
 - The CSM mix you use should be appropriate for the experiments you plan; CSM mix can contain all amino acids, or with particular amino acids removed (single, double, and triple dropout)
- [Yeast Nitrogen Base \(YNB\) with Ammonium Sulfate w/o Amino Acids](#)
- 10 g agar
- Stir bar
- 1000 mL Erlenmeyer flask
- 470 mL distilled H₂O (no need for Milli-Q water, but do not use tap water)
- Petri (cell-culture) dishes

Protocol

1. Mix the following in a 1000 mL Erlenmeyer (conical) flask
 - 0.305 g CSM mix
 - 3.3 g Yeast Nitrogen Base with Ammonium Sulfate w/o Amino Acids
 - 10 g agar
 - Stir bar
 - 470 mL distilled H₂O
2. Autoclave for 20 min.
3. After autoclaving, add 25 mL 40% glucose solution and place on a stirrer for a slow, gentle stir
4. **Add-back nutrients** – add **liquid stocks** post-autoclaving ONLY (no powders)
 - See Table A1 in “Methods in Yeast Genetics” for info on making stock solutions.
5. Let stand for 20-30 min on a stirrer until the flask cool enough to touch, then pour the plates.

Liquid CSM (filter-sterilized version, 500 mL)

Materials

- CSM mix
- Add-back nutrients
- Glucose

Protocol

1. In a graduated cylinder, mix the following:
 - ~0.325 g CSM mix
 - [See product label for exact amount.](#)
 - Add-back nutrients (50 mg Leu, 10 mg Ura)
 - 3.3 g Yeast Nitrogen Base with Ammonium Sulfate w/o Amino Acids

- 10 g Glucose (Dextrose)
- 2. Add distilled H₂O to 500 mL
- 3. Transfer to a beaker, add a stir bar, and wait for powders to dissolve.
- 4. Filter-sterilize, preferably with a bunsen burner around.

Yeast inoculation

1. Heat inoculating loop
- 2.
3. 4 mL YPD in culture tube
4. 30°C, 280 RPM, 16 hours

Preparing selection media

All drugs are temperature sensitive, so you need to add them only **after your autoclaved solution has sufficiently cooled down** (enough to touch the flask for 5+ seconds with a gloved hand without getting burned, or equivalently after about ~20-45 min outside on a stir plate).

G418 / Hygromycin / clonNAT (Nourseothricin) (500 mL)

- For YPD-based media: follow the usual protocol and add drugs once sufficiently cooled
 - Drugs are temperature-sensitive
- For CSM-based media there is an extra adjustment:
 - **Do NOT add 3.3 g YNB**
 - Instead, add 0.85 g YNB w/o Ammonium Sulfate and Amino Acids
 - Add 0.5 g Glutamic acid and monosodium salt hydrate (MSG)
 - This is done because all three drugs are not effective in media containing ammonium sulfate, which we substitute with MSG as above
- Let stand for 30-60 min, while stirring, until flask cool enough to touch, then add one or a combination of the following:
 - 1 mL G418 aliquot (100 mg/mL)
 - 0.5 mL clonNAT aliquot (100 mg/mL)
 - 3 mL Hygromycin aliquot (50 mg/mL)

LB+Amp (500 mL)

- 20 g LB Agar Mix
- Add DI H₂O to 500 mL, add a stir bar
- Autoclave 20 min
- Stir and let solution cool ~20-30 min (ideally, to near-room temperature)
- Add 500 µL 1000x (=100 mg/mL) Amp stock (stored at -20 C)
 - Ampicillin is temperature-sensitive

IDT Primer resuspension

- Briefly centrifuge
- Resuspend in $10 \times (\text{nmol supplied}) \mu\text{L}$ of EB buffer, for a $100 \mu\text{M}$ stock
- For aliquots, dilute 1:10 in distilled H_2O (e.g. $5 \mu\text{L}$ in $45 \mu\text{L}$ H_2O)

Preparing Liquid Amino Acid Stock Solutions (50 mL)

Materials (per amino acid stock solution)

- Amino acid, as dry powder
- One 150 mL beaker

Protocol

Constituent	Stock concentration (g/100 ml)	Volume of stock for 1 liter of medium (ml)	Final concentration in medium (mg/liter)	Volume of stock to spread on plate (ml)
Adenine sulfate	0.2 ^a	10	20	0.2
Uracil	0.2 ^a	10	20	0.2
L-Tryptophan	1	2	20	0.1
L-Histidine HCl	1	2	20	0.1
L-Arginine HCl	1	2	20	0.1
L-Methionine	1	2	20	0.1
L-Tyrosine	0.2	15	30	0.2
L-Leucine	1	10	100	0.1
L-Isoleucine	1	3	30	0.1
L-Lysine HCl	1	3	30	0.1
L-Phenylalanine	1 ^a	5	50	0.1
L-Glutamic acid	1 ^a	10	100	0.2
L-Aspartic acid	1 ^{a,b}	10	100	0.2
L-Valine	3	5	150	0.1
L-Threonine	4 ^{a,b}	5	200	0.1
L-Serine	8	5	400	0.1

^aStore at room temperature.

^bAdd after autoclaving the medium.

1. Using column 1 ("Stock concentration [g/100 ml]"), add **half** the number of grams listed to **50 mL** Milli-Q water in a 150 mL beaker.
2. Stir until amino acid is completely dissolved.
3. As amino acids can be damaged by high temperatures, **filter-sterilize** the solution into a 50 mL conical (Falcon) tube.

Preparing glycerol stocks (2 mL)

- Dilute saturated liquid culture with 50% glycerol in 1:1 ratio

Eurofins Sequencing

- 4 μL Primer (10 μM)
- x μL of 0.5-1.2 μg total DNA
- UltraPure H₂O -> to 12 μL total

Strain Collections (KO, FLEXGene, etc.)

- Het- and Hom- Diploid strains (KanMX) can go on YPD or G418 plates
- KO (a and alpha) haploid strains can go on YPD or G418
- GFP strains can go on YPD or CSM-His
- MOBY 2.0 (KanMX) strains can go onto YPD or CSM(MSG)-Leu+G418
- FLEXGene (pBY011 pGAL- vector) can go on CSM-Ura

Yeast KO I - PCR + Gel Electrophoresis

<https://redrecombineering.ncifcrf.gov/protocols/step-by-step-knockout-dsna.pdf>

Time: 4-5 hours

1. Make a PCR Master Mix (everything you will add to the rxn minus DNA):

For two PCR reactions, adapted from [here](#), pay attention to concentrations!

Note: if plasmid is used as a PCR template, you must perform a restriction digest first to linearize it

Stock Component	Volume "Master Mix"	Volume/reaction	Final concentration/amount in rxn
10X Polymerase Buffer	12.5 uL	5 uL	1X
10 mM dNTP mix	3.75 uL	1.5 uL	300 uM each
Forward primer (5uM stock)	12.5 uL	5 uL (25-50 pmols)	0.5 uM
Reverse primer (5 uM stock)	12.5 uL	5 uL (25-50 pmols)	0.5 uM
Taq Polymerase	1.25 uL	0.5 uL	2.5 units
H2O	76.25 uL	30.5 uL	
Template DNA (20 ng/uL)	(none)	2.5 uL	50 ng

2. Mix contents of Master Mix by vortexing or flicking.

3. Pipet 47.5 uL of Master Mix into two *labeled* PCR tubes

4. Add 2.5 uL of DNA template(s) to the relevant tube(s)

5. Transfer the tubes to a thermocycler and run the following program:

(94 C, 3 min; 42 C, 1 min; 72 C, 1 min 10 sec) x2, (94 C, 30 sec; 56 C, 30 sec; 72 C, 1 min 10 sec) x28, (72 C, 5-10 min) x1, (4 C, hold)

6. Add loading dye to 5 uL of each PCR product and run on agarose gel with a DNA ladder

See sections about "Pouring Gels" + "Gel Electrophoresis"

Notes: our template DNA will be a plasmid that contains a deletion module (e.g. kanMX); what PCR does here is it adds homology arms to the ends of the deletion module that will be used for

HR into the yeast genome at the site of our target gene that we want to KO (no need to clean-up PCR product).

After transformation + selection (+ genetic DNA isolation), integration and deletion is analyzed by PCR (using our pre-made verification primers) and gel electrophoresis

I'm assuming verification PCR is basically identical to the above protocol, but will check that