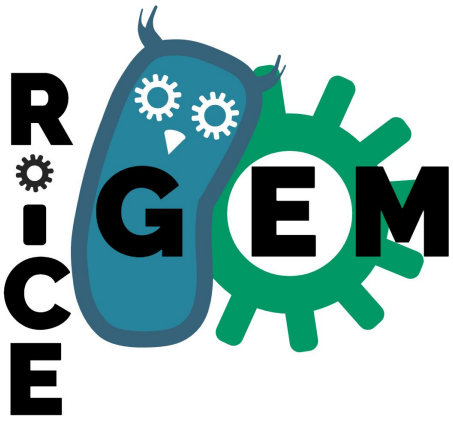

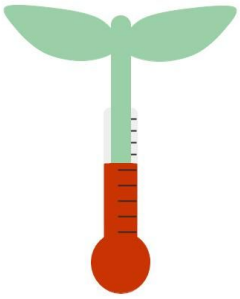

Thermoplant

Standard Protocols

Rice University

2019 iGEM Team

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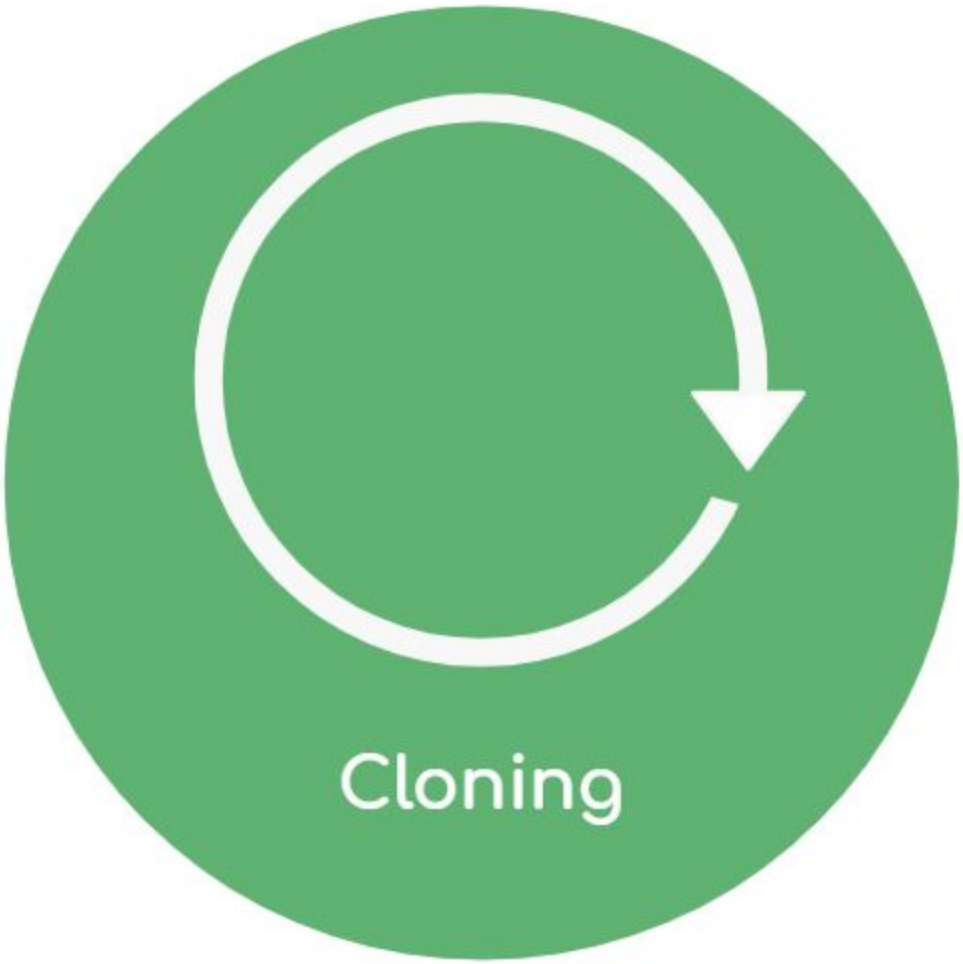
Cloning

LB30 Media Preparation

Materials

- Yeast Extract
- Tryptone
- NaCl

Procedure

- Per 1 L, combine 30 g of yeast extract, 10 g of Tryptone and 10 g of NaCL
- add one half of water into bottle, then the solids, then add water until you get to 1 L final volume
- expect the color to be significantly darker than normal LB
- Autoclave on Liquid 15 protocol
- Use aseptic technique when drawing from LB30. Take particular care to flame both the lid and rim prior and after using.
- Can typically get the same final concentration of DNA in a miniprep with ⅓ the volume as you would for LB
    - so if 6 mL of LB is used, 2 mL of LB30 can be substituted.
    - This is what makes LB30 preferable to LB. You can grow in 24 well blocks that only hold about 2 mL, Transfer to 2 mL tube, pellet, resuspend and lyse all in the same tube.

LB Plate Preparation

Materials

- Yeast Extract
- Tryptone
- NaCl
- Agar
- antibiotic
- plates

Procedure

- Per 1 L, combine 5g of yeast extract, 10g of Tryptone, 10g of NaCL and 20g of Agar
- add one half of water into bottle, then the solids, then add water until you get to 1L final volume
- expect the color to be significantly darker than normal LB
- Autoclave on Liquid 15 protocol
- Cool till <50˚C before adding the appropriate amount of antibiotic.
    - to make cooling faster, we recommend adding a stir bar before autoclaving, putting the media in a water bath on to of a magnetic shaker
- Pour or use a serological pipette to aliquot into the plates
- briefly flame top in event of bubbles
- let dry for 24 hours closed in room temp before using, then store at 4˚C

Inoculating a Liquid Bacterial Culture

Materials

- Bunsen burner (or ethanol candle)
- LB broth
- 1000X antibiotic (chloramphenicol (Chl) or ampicillin (amp))
- 50 mL centrifuge tubes (SN C-3394-3 ISC BioExpress)
- LB agar plate with antibiotic resistant bacteria
- Shaking incubator (SN 06004817 VWR)
- 14 mL cell culture tubes (SN 07418023 Falcon)
- 1.5 mL centrifuge tubes

Procedure

- Label *n* number of 14 mL polypropylene round-bottom culture tubes as needed with the plasmid number
- Pipette 2*n* mL LB agar into 50 mL centrifuge tube using aseptic technique
    - Note: n = the number of tubes labeled in step 1
- Add 2*n* µL 1000X antibiotic (Chl or amp) to the LB broth
- Aliquot 2 mL of the LB broth + antibiotic into each 14 mL culture tube
- Use a micropipette and sterile pipette tip to select a single colony from the LB agar plate
- Eject the pipette tip into the prepared media of LB + antibiotic and swirl
- Loosely cover the culture tube with a cap
- Incubate the culture at 37 ˚C in a shaking incubator for 12-18 hr
- Store the bacteria plate in the 4 ˚C fridge
- After incubation, pipette 1.5 mL of each liquid culture into 2 1.5 mL tubes labeled with the plasmid number
- Spin down at 6000 rcf for 1-3 min
- Remove supernatant by dumping it into a liquid waste container
- Store the remaining pellet in -20˚ C freezer or proceed to DNA miniprep

Oligonucleotide Annealing

Materials

- Phosphorylated oligos
- 0.2 mL PCR tubes
- Nuclease free water
- Thermocycler
- Centrifuge

Procedure

1. Dissolve the oligos to a final volume of 100 µM
2. Add 10µL from each of the tubes into a new PCR tube
3. Add 80µL of nuclease free water, making the final reaction volume in the PCR tube = 100µL
4. Use the following thermal profile:
    a. Heat to 95 ˚C and maintain the temperature for 2 min.
    b. Cool to 25 ˚C over 45 min (or in 0.1 ˚C)
    c. Cool to 4 ˚C for temporary storage.
5. Centrifuge the PCR tube briefly to draw all moisture away from the lid

Oligonucleotide Phosphorylation

Materials

- 10X T4 ligase buffer
- T4 PNK
- Nuclease-free water
- Oligo anneal product

Table 1: Oligo Phosphorylation materials

Component	Volume
Annealing product	0.5 µL
10X T4 Ligase buffer	2.5 µL
T4 PNK	0.5 µL
Nuclease-free water	to 25 µL (21.5 µL)

Procedure

1. For each oligo anneal product, make a 25 µL reaction with the components listed in Table 1
2. Incubate for 30 min at 37 ˚C

Cloning PCR

Materials

- Thermocycler
- 0.2 mL PCR tubes
- Ice or cold block
- Components in Table 1

Table 1: Cloning PCR materials

Component	Volume	Final Concentration
2X Phusion PCR master mix	12.5 µL	1X
10 µM Forward Primer or 100 µM	1.25 µL or 0.125 µL	0.5 µM
10 µM Reverse Primer or 100 µM	1.25 µL or 0.125 µL	0.5 µM
Template DNA	Variable ≈ 1 µL	1 ng - 1 pg plasmid/viral 1 ng - 1 µg genomic
Nuclease-free water	to 25 µL	1.0 units per reaction

Procedure

1. Centrifuge primers for 30 sec at 10,000 rcf
2. Make 10x dilutions of 100 µM primers and 100x dilution of 20 ng/µL template
3. Vortex primers for 10 sec and centrifuge again for 30 sec at 10,000 rcf
4. Assemble 25 µL reactions on ice in PCR tubes as in Table 1
5. Close tubes, flick tubes a few times to mix, and centrifuge a few seconds to recollect the liquid
6. Thermocycle on pre-heated block using conditions in Table 2
    a. we are generally removed the final holding temperature to preserve the thermomocycler

Table 2: Cloning PCR thermocycling conditions

	Step	Temp	Time	Notes
	Initial Denaturation	98 ˚C	30 s - 3 min	30 s for most templates (plasmid/linear/E. coli). 1-3min for complex, or to better lyse cells.
25-35 cycles (30-35 genomic)	Denaturation	98 ˚C	5-10 s	*Find T anl, 3 ˚C above Tm of lower Tm primer
	Annealing	50-72 ˚C	10-30 s	10-15 s/kb for simple plasmid/E. coli template, or <1 kb complex template
	Extension	72 ˚C	10-40 s / kb	
	Final Extension	72 ˚C	2 min	

Colony PCR of thermometers

Materials

- Thermocycler
- 0.2 mL PCR tubes
- Ice or cold block
- Components in Table 1
- saturated cell culture

Table 1: Colony PCR materials

Component	Volume	Final Concentration
Taq DNA polymerase	0.08 µL	0.025 U/µL rxn
10x Taq reaction buffer	1.5 µL	1x
10mM dNTPs	0.3µL	200µM
10 µM Forward Primer for each RNA thermometer or 100 µM	1.25 µL or 0.125 µL	0.5 µM
10 µM Reverse Primer (AD65) or 100 µM	1.25 µL or 0.125 µL	0.5 µM
Cell Culture	Variable ≈ 5 µL	1 ng - 1 pg plasmid/viral 1 ng - 1 µg genomic
Nuclease-free water	to 15 µL	1.0 units per reaction

Procedure

1. PCR is always set up at 1˚C
2. Centrifuge primers for 30 sec at 10,000 rcf
3. Make 10x dilutions of 100 µM primers and 100x dilution of 20 ng/µL template
4. Vortex primers for 10 sec and centrifuge again for 30 sec at 10,000 rcf
5. Assemble 25 µL reactions on ice in PCR tubes as in Table 1
6. Close tubes, flick tubes a few times to mix, and centrifuge a few seconds to recollect the liquid
7. Thermocycle on pre-heated block using conditions in Table 2

Table 2: Colony PCR thermocycling conditions

	Step	Temp	Time	Notes
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25-35 cycles (30-35 genomic)	Denaturation	98 ˚C	10 s	*Find T anl, 3 ˚C above Tm of lower Tm primer
	Annealing	50-60 ˚C	30 s	10-15 s/kb for simple plasmid/E. coli template, or <1 kb complex template. Annealing temperatures are checked using NEB Tm calculator. Taq typically requires a lower annealing temperature than Q5.
	Extension	72 ˚C	30 s / kb	
	Final Extension	72 ˚C	5 min	

Golden Gate Assembly

Materials

- 0.2 mL PCR tubes
- Ice or cold block
- Thermocycler
- Components in Table 1

Table 1: Golden Gate reaction materials

Component	Volume	Notes
BsaI/Esp3I/BbsI	0.5 µL	0.5–0.75 µL range
T_4 DNA Ligase	0.5 µL	0.5–1 µL range, 2000 CEU/µL cligase ∝ misligation
10X T_4 Ligase Buffer	1.5 µL	Titurate/vortex to dissolve solids Limit freeze/thaws to ~5-10x
10X BSA + PEG	1.5 µL	10x: 1mg/mL BSA, 10% PEG-3350 BSA: Enables full BsaI activity at 37˚C, absorbs contaminants PEG: reduces diffusion, enhancing ligation efficiency
DNAs	25 fmol each 0.5 µL 50 nM	10–40 fmol equimolar 2–5-fold less vector to reduce background
DI water	Up to 15 µL	10-20 µL range

Procedure

1. Dilute samples to 50 nM
2. Prepare a master mix on ice or cold block by adding together enzyme, T_4 DNA Ligase, 10X T_4 Ligase Buffer, 10X BSA, and water.
3. Pipette up and down to mix
4. Distribute mastermix into labelled PCR tubes on ice or cold block
5. Add DNA components for each reaction to the concentrations specified in Table 1
6. Flick to mix
7. Centrifuge for a few seconds to recollect the liquid
8. Place the reaction tubes into the thermocycler and adjust the settings for a given enzyme as shown in the following tables

Table 2: Esp31 Golden Gate assembly thermocycling conditions

Long, ≥ 6 parts: 142.5 min / 2:23; Short, ≤5 parts: 97.5 min / 1:38					**Basic, 2-3parts: 52.5-75min**		
Notes	Step	Temp	Time		Notes	Temp	Time
	Initial Digestion (opt.)	37˚C	10 min			37˚C	20 min
Repeat 25x/15x	Digestion	37˚C	1.5 min		Repeat 5-10x	37˚C	1.5 min
	Annealing & Ligation	16˚C	3 min		Repeat 5-10x	16˚C	3 min
	Digestion	37˚C	5 min			37˚C	5 min
	Digestion & Ligase Inactivation	50˚C	5 min			50˚C	5 min
	Inactivation	80˚C	10 min			80˚C	10 min
	Storage	12˚C	∞			12˚C	∞

Table 3: Bsal Golden Gate assembly thermocycling conditions

Long, ≥ 6 parts: 142.5 min / 2:23; Short, ≤5 parts: 97.5 min / 1:38					Basic, 2-3parts: 52.5-75min		
Notes	Step	Temp	Time		Notes	Temp	Time
	Initial Digestion (opt.)	37˚C	10 min			37˚C	20 min
Repeat 25x/15x	Digestion	37˚C	1.5 min		Repeat 5-10x	37˚C	1.5 min
Repeat 25x/15x	Annealing & Ligation	16˚C	3 min		Repeat 5-10x	16˚C	3 min
	Digestion & Ligase Inact.	50˚C	10 min			50˚C	10 min
	Inactivation	80˚C	10 min			80˚C	10 min
	Storage	12˚C	∞			12˚C	∞

E. coli Competent Cell Preparation

Materials

- Transformation/storage solution (TSS) buffer
- Sterile rich growth medium (+antibiotic if necessary) (e.g LB broth)
- Culture tubes for pre cultures
- Culture flasks large enough to hold growth medium volume (detergent-free).
    - 250 mL flask usually
- Shaking incubator space for the culture flasks
- Ice bucket/tray large enough to swirl flasks in. Access to ice.
- Chilled centrifuge bottles/lids or tubes appropriate for holding culture volumes and balancing. (detergent-free)
    - Bottles must be able to collectively hold culture(s) without any exceeding ¾ capacity. Filling bottles higher will result in leakage into rotor.
- Chilled sterile water to balance multiple centrifuge bottles, if needed.
- 4˚C centrifuge and rotor compatible with centrifuge bottles.
- (*opt.*) A serological pipette at -20˚C for adding *nv* volume TSS before going to cold room.
- Labeled cryoboxes for comp cell aliquots, prechilled at -80˚C.
- (*opt.*) Liquid nitrogen, dewar, and slotted ladle, if flash freezing.
- Materials on a mobile lab bench cart for the cold room:
    - *n* tubes (e.g. 200 µL tubes) for cell aliquots, labeled/marked and arranged in clean tube racks or tip rack+boxes. Blade, if planning to cut apart tube strips.
    - Extra tubes or bag of tubes, for aliquoting any excess comp cells, if desired.
    - Serological pipette pump/controller and few 20 mL pipettes, for resuspending and transferring cells.
    - (*opt.*) Electronic repeater pipette and a 5 mL combitip per strain and some extras. Reservoirs, if opting to us a multichannel pipette.
    - A 50 mL tube per strain to hold resuspension.
    - Micropipette and tips appropriate for aliquot volumes ≥*v*. (Sometimes useful to aliquot remaining volume even when using electronic repeater pipette.)
    - Ethanol spray
    - Paper towels
    - Trash bin/bag
    - (*opt.*) If flash freezing, box/bag to collect all tubes in before flash-freezing.

Procedure

1. Grow preculture to saturation: inoculate ≥≈1/100 culture volume of rich medium (+selection, if necessary) with colony or frozen stock chunk, and incubate at growth temp (*e.g.* 37 ˚C) shaking for ≥≈8 hr for LB
    a. *Use a fresh, trusted source of the strain. For the most reliable results, inoculate a seed culture from colonies from relatively fresh plates streaked from a frozen stock. Don't obtain from old plates; retransform necessary plasmid(s) or obtain directly from a frozen stock*
2. Meanwhile, prepare growth medium and warm to growth temperature
3. Prepare the mobile lab bench cart for the cold room as described
4. Inoculate 20*nv* mL pre-warmed LB with ≈0.2% seed culture (or ≈7*nv* mL TB/LB^{30} with same)
5. Incubate 37˚C, shaking 250 rpm for flasks or 800 rpm for blocks
6. Grow LB culture to OD_{600}≈0.2–0.35 (early exponential phase). For TB/LB^{30} OD_{600}≈1–2. It requires 1.5–3 hours at 37˚C, 250 rpm in a large flask, a bit slower at 30˚C. Monitor with periodic OD measurement
7. In the meantime, prechill both the mobile lab bench cart (with its contents) and the centrifuge to 4˚C ≥45 min before cell harvest (next step).
8. When in early log phase (OD_{600}≈0.2–0.35), swirl culture vessel in ice water bath for several minutes *until 4˚C* to rapidly stop growth. It takes up to 20 min for ~1 L volumes
9. When culture is ice-cold, decant into prechilled centrifuge bottles and balance them. Use chilled sterile water if necessary
10. Centrifuge cells 2500×*g*, 5–20 min (depending on volume) in a prechilled 4˚C centrifuge
11. Check for a small pellet. Gently decant medium away from pellet, shaking the bottle to drain. Absorb the last of the medium on the lip with a paper towel. Return bottles to ice
12. Perform remaining steps with pre-chilled materials in the cold room for maximum efficiency
13. To the cell pellets, add 5% of the original culture volume of 4˚C TSS (*nv* mL). For TB/LB^{30} culture, 14% volume TSS.
14. *Gently* resuspend pellet in TSS using serological pipette, or P1000 tip if small volume
15. For large volumes, decant cells into 5 or 50 mL conical tubes on ice for easier access with pipette for aliquoting
    a. *Flat-bottom centrifuge bottles allow easier gentle resuspension of pellets by pipetting against wall*
    b. *Original protocol instructs 1–10-fold concentration in TSS. 20-fold as here, or even 40-fold increases transformation efficiency, but requires larger culture with diminishing returns in efficiency*
16. Aliquot into tubes in the cold room (for top efficiency) or on cold block or ice. You can use a repeater pipette or multichannel pipette from a chilled reservoir

17. Cap the tubes while they are racked, and slice apart if using tube strips. Minimize touching the tubes to keep them cool. Check that all caps are fully in the tube
18. Dislodge tubes from racks into a bag/box without touching the bottom (your hands are warm). This is easiest by pushing them out from the bottom using a spare rack or tip box
19. (*opt.*) Flash-freeze in liquid nitrogen, supposedly for maximum efficiency.
    a. *Slow freezing works well, too, maybe even better. You can likely omit this step*
    b. *Residual ethanol from a dry-ice-ethanol bath fails to dry from tubes/boxes at -80˚C. Flash-freeze tubes in a bag if using this method, and pour tubes into box*
20. Quickly move tubes to prechilled, labeled -80˚C freezer boxes, either directly from 4˚C, or if flash freezing, directly ladled out of liquid nitrogen dewar or dry-ice/ethanol bag (so as not to heat tubes)
21. If not freezing, proceed to transformation right away

P. putida Competent Cell Preparation

Materials

- *P. putida* culture
- Standard 1-medium (growth medium)
- 10% glycerol washing solution
- 10% glycerol electroporation solution

Procedure

1. Inoculate 50 mL standard-1 medium with 7 mL of a fresh overnight culture of *P. putida*. Grow cells at 30 deg C to a density of O.D. of 0.8
2. Harvest by centrifugation
3. Wash twice with 50 mL ice-cold glycerol, centrifuge
4. Resuspend cells in 0.8 mL ice-cold glycerol, keep on ice
5. Aliquot into PCR tubes

TSS Heat Shock Transformation

Materials

- Comp cell aliquots (aliquot standard 50 µL)
- DNA
- Ice or cold block
- Heated block, water bath or thermocycler
- Recovery medium (e.g. SOC)
- Incubator
- LB agar plate with antibiotic

Procedure

1. Thaw -80˚C comp cell aliquots on ice or cold block for 5 min (or use comp cells within hours of comp cell prep). Keep at 4˚C except for heat shock and recovery
2. Add DNA, aiming for ≤10% comp cell volume
    a. We add 5 µL to a 50 µL aliquot for golden gates and 0.5µL for retransformations
    b. For transformations that we expect to have a low efficiency, like 7 part golden gates or older comp cells, we used 2 -3x the amount of comp cells. We achieved this by thawing the comp cells and transferring the comp cells from one tube to another labeled tube and then adding the DNA.
3. Flick five times to mix. Do not vortex or triturate
4. Incubate at 4 ˚C (ice or cold block) for 5–30 min
    a. We do 30 min
5. Heat shock
    a. For E. coli Turbo heat shock at 30˚C for 30 sec
    b. For E. coli DHB10 alt heat shock at 42˚C for 45 sec
6. Immediately return to 4˚C (ice or cold block)
    a. For E. coli Turbo heat shock at 30˚C for 30 sec
    b. For E. coli DHB10 alt heat shock at 42˚C for 45 sec
7. For E. coli Turbo, we advise repeating the heat shock and ice recovery
8. Remove from 4˚C. Add 1–10 volumes room-temp recovery medium (non-selective). Flick to mix (trituation or gentle vortexing may be ok).
    a. We add 3 volumes (150 µL) SOC to a 50 µL comp cell aliquot
9. Incubate at growth temp (often 37˚C) for 45–90 min, optionally shaking.
    a. We do 60 min, no shaking
10. Spread/streak a fraction of the transformation on selective agar medium (plates), optimally room-temp or warmed to growth temperature (often 37˚C).

   a. Store the remainder at 4˚C in case later needed due to finding too few or too dense colonies on the transformation plate.
   b. Spread or streak only as much as can fit on the plate/sector without either flowing into neighboring sectors or inhibiting streaking dilution such that single colonies are not obtainable (the case with higher efficiency transformations).
11. Incubate plates for 12-15 hr at growth temperature (often 37 ˚C)

P. putida Electrotransformation

Materials

- DNA samples
- *P. putida* electrocompetent cells
- Cuvettes
- Antibiotic selective plates

Procedure

1. Pipette 4 µL plasmid DNA in to 40 µL *P. putida* electrocompetent cells. Homogenize by gently mixing with pipette several times
2. Transfer mixture into a the middle of prechilled cuvettes
3. Gently tap the cuvette on the table
4. Wipe moisture along the sides of the cuvette and insert cuvette into the device, making sure to align it to the device notch
5. Electroporation
    a. 1200 V for 1 mm cuvette
6. Immediately add 1 mL standard 1-medium
7. Incubate 2 hrs at 30 ˚C
8. Plate cells on selective plates

DNA Miniprep

Materials

- QIAprep Spin Miniprep Kit
- 1-6 mL saturated bacterial cultures
- 1.5 mL centrifuge tubes
- Vortex
- Centrifuge

Procedure*

1. Pellet 1-6 mL saturated cultures by centrifugation at 6000 rcf for 3 min
2. Resuspend the pelleted cells in 250 µL of Buffer P1
    a. Store Buffer P1 in the 4˚ C fridge
3. Vortex the resuspended cells for 30 seconds
4. Add 250 µL Buffer P2 to each tube and mix by inverting 4-6 times until the solution becomes clear
    a. Don't let sit for more than 2 min
5. Add 350 µL Buffer N3 to each tube and mix by inverting 4-6 times
6. Centrifuge for 10 min at 16,100 rcf
7. Pipette 800 µL of the supernatant into a spin column
8. Centrifuge the spin columns for 1 min at 16,100 rcf and discard flowthrough
9. (opt.) Add 500 µL Buffer PB to each column for strains with high-carbohydrate or high nuclease activity. Centrifuge the columns for 1 min at 16,100 rcf and discard flowthrough
10. Add 750 µL Buffer PE to each column to wash
11. Centrifuge for 1 min at 16,100 rcf and discard the flowthrough
12. Place 1 mL Buffer EB in a 37˚ C incubator
13. Label 1.5 mL centrifuge tubes with the plasmid numbers and transfer the columns to the 1.5 mL tubes
14. Dry spin the columns for 1 min at 16,100 rcf to remove residual wash buffer
15. Add 50 µL Buffer EB to each column to elute DNA and let stand for 5 min
    a. Add 30 µL instead of 50 µL if using a low copy vector to get better concentrations
16. Centrifuge for 1 min at 16,100 rcf
17. Remove the spin column from tubes
18. Use nanodrop spectrophotometer to check DNA concentration
19. Store in -20˚ C freezer or proceed to analyze extracted DNA on a gel by adding the appropriate amount of loading dye

**We reference and modify the Qiagen protocol found in the kit*

NanoDrop Spectrophotometry

Materials

- NanoDrop Spectrophotometer
- Kimwipes
- Miniprepped samples

Procedure

1. Lift the NanoDrop arm and clean with ethanol and wipe with a Kimwipe
2. Once cleaned, dispense 1.5 µL of blanking solution directly on top of the NanoDrop sensor
3. Lower the arm and click the “Blank” button
4. Once the blank is complete, lift the NanoDrop arm and use a Kimwipe to clean the sensor and metal cap located on the arm
5. Make sure that the sample to be read is properly suspended. Add 1.5 µL of the sample onto the NanoDrop sensor, close the arm, and click “Measure”.
6. Record the value (in ng/µL).
7. Take two measurements of each sample, and make sure the NanoDrop sensor and metal cap are cleaned with a Kimwipe after each sample.

Restriction Digest

Materials

- Restriction enzyme
- Plasmid DNA
- 10X restriction digest buffer
- 0.2 mL PCR tubes

Procedure

1. Mix, by pipetting up and down, 1µL 10X buffer, and 7.5 µL water
2. Add in 0.5 µL of enzyme, and mix by pipetting up and down
3. Mix in 200 ng of template DNA
4. Incubate samples for 30-60 min at 37 ˚C
    a. NotI is suggested to run for 30 min
5. Store samples at -20 ˚C or use for gel electrophoresis

Agarose Gel Preparation

Materials

- Scale
- Microwavable flask
- Agarose powder
- 1X TAE
- Microwave
- Silicone mitt
- 10,000X DNA dye (GelGreen or GelRed)
- Gel tray(s)
- Combs of appropriate thickness

Procedure

1. Weigh and mix agarose powder with 1X TAE to desired agarose concentration in a microwavable flask or bottle
    a. Example: For a 1% gel, use 1 g agarose with 100 mL 1X TAE
2. With a loosened cap, microwave agarose suspension or solid until completely clear and homogeneous liquid (≈1 min per 100 mL on high).
    a. Watch as it boils to stop the microwave before it can overflow. Swirl, handling with a silicone mitt. Continue microwaving in smaller increments if necessary.
3. If prestaining the gel, to a final 1X, add 10,000X DNA dye (GelGreen or GelRed) to the molten agarose and swirl until color is uniform
    a. Example: Add 10 µL to 100 mL molten agarose
4. Position tray(s) in the center of clamp and tighten clamp moderately. Place desired comb(s) in tray slots, oriented so the prongs are closer to the top of the gel.
5. Ensure tray is level using bubble level, adjusting gel caster's screw feet. Pick gel casting trays appropriate for your use.
    a. The 7 cm tray holds 40 mL when filled to the top of comb prongs
    b. The 10 cm tray holds 55 mL when filled to the top of comb prongs
    c. For gel purification purposes, you may want a thick gel (tray filled to top of comb prongs) to minimize the number of wells needed to hold the samples.
    d. For analytical purposes, you may need only a thin gel (casted to half prong height) to hold small volumes of samples such as analytical DNA digests.
    e. Pick comb appropriate for your use.
        i. Thinner combs are superior for analytical purposes, as they result in thin bands more accurately assessable.

   ii. Thicker combs can be superior for purification purposes, as they hold more sample. A full-height narrow well holds ≈30 µL; a full-height wide well holds ≈60 µL.
6. Pour molten agarose in trays to desired level while still hot, but not boiling to prevent tray/clamp warping. Use comb to pull bubbles and impurities away to end of gel. Bubbles can be pushed to the edges of the gel using the comb.
7. Allow the gel to solidify at room temperature for 20-30 min or level at 4˚C, 8–15 min. Pre-chilled caster and tray speeds solidification.

Loading Samples and Gel Electrophoresis

Materials

- Prepared agarose gel
- DNA samples
- Loading buffer
- Pre-dyed DNA ladder
- Electrophoresis chambers
- Power supply
- Blue light and orange glasses OR UV light and UV shield

Procedure

1. Add loading buffer to each sample
    a. If sample-staining, ensure the loading dye contains 1X GelGreen/GelRed. Loading dye does not typically contain any DNA dye, only tracking dyes that allow you to see your sample when loading and visibly estimate DNA migration progression. Loading dye also contains glycerol, sucrose, or PEG to weigh down the sample in the well.
2. Fill electrophoresis chamber with 1X TAE (running buffer) until it's a bit under the gel + tray height.
    a. If running buffer is cloudy or has crystals, empty contents into gel liquid waste tank. Gently rinse chamber in sink without pointing stream at deep ends, where it can damage the electrode filaments. Do a final rinse with diH2O. Refill with 1X TAE. When using ethidium bromide, some people add it to the running buffer as well as the gel.
3. Remove comb from cast gel and submerge gel+tray in the electrophoresis chamber, oriented with wells at the top and the negative (black) terminals of the chamber at the top.
    a. If TAE doesn't cover the gel, add sufficient TAE. Gels can be cut to save lanes for later use. The tray isn't always necessary. Gels must be stored in TAE + DNA dye (if prestained), TAE (if unstained), or wrapped ≈airtight in plastic wrap.
4. Load samples and, finally, ladder into wells. Pre-dyed ladder is generally at the gel station.
    a. While loading, maintain positive pressure on the sample to prevent bubbles or buffer from entering the tip. Release any air at the tip of the tip so no bubbles are present, since it is likely to cause the sample to be released

when loading. Hover the tip of the tip above the well. Slowly and steadily, push the sample out and watch as the sample fills the well. After all of the sample is unloaded, push the pipettor to the second stop as you raise the pipette out of the buffer. Pipetting into the bottom of the well (a more difficult method) allows more sample to fit in a well. Moving the gel or pipette tip while it's in a well containing sample may expel part of the sample from the well. Keep everything steady when ejecting inside a well.

5. Secure the lid onto the chamber, and plug in lid into power supply. Match terminal colors. Ensure negative black terminals are at the top of the chamber and lid and positive red at the bottom. Thus anionic nucleic acids run to the red anode. Run to red. Program in voltage (5–10 V/cm electrode distance) and run time and start. Confirm running by spotting bubbles emanating from the electrodes.
    a. Common settings are 80–120–150 V, 60–30–15 min. Run time is inversely correlated with voltage.
6. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
7. (opt.) If poststaining, stain the gel in TAE + 1–3× DNA dye solution, rocking for 15–30 min, followed by destaining in water if necessary for sensitivity.
8. Inspect/cut the gel under blue light with orange glasses or UV with UV shield
    a. Image the gel with the blue light (or UV) imager: Open Canon EOS utility on the computer to awaken the connected camera and use Remote Live View to adjust the gel position and autofocus before taking a picture. The camera should typically be in the No Flash setting (auto-set exposure, ISO, and aperture). Exit EOS Utility when done. Blue light is safer to work with and less damaging to DNA than UV light. Requires GelGreen, SYBR green, SYBR Safe, etc.
    b. Use the DNA ladder as a guide to analyze your gel and interpret the bands as expected or not

Gel Purification

Source:
https://files.zymoresearch.com/protocols/_D4001T_D4001_D4002_D4007_D4008_Zymoclean_Gel_DNA_Recovery_Kit_ver_1_2_1_LKN-SW_.pdf

Materials

- Agarose gel with samples
- Zymoclean DNA Gel Recovery Kit
- 1.5 mL centrifuge tubes
- Razor
- Scale
- Incubator
- Centrifuge

Procedure

1. Cut out bands for all sample from agarose gel using a razor and transfer each into different 1.5 mL centrifuge tubes
    a. Try to cut as little agarose as possible
2. Weigh, in milligrams (mg), an empty 1.5 mL centrifuge tube and then weigh the 1.5 mL centrifuge tube with the gel. Subtract the empty tube weight from the tube weight with the gel to obtain the weight of the gel itself. Multiply this weight by 3.
3. Add 3 volumes of agarose dissolving buffer (ADB) to each tube
    a. (e.g. for 100 µL (mg) of agarose gel slice add 300 µl of ADB)
4. Incubate at 37-55 ˚C for 5-10 minutes until the gel slice is completely melted
    a. Gentle mixing may be used to facilitate melting
5. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
6. Centrifuge for 30-60 seconds. Discard the flow-through by aspiration
7. Add 200 µL of DNA Wash Buffer to the column and centrifuge for 30 seconds.
8. Discard the flow-through. Repeat the wash step.
9. Dry spin for 2 min to remove residual wash buffer
10. Add ≥ 6 µL DNA Elution Buffer or water directly to the column matrix
    a. We use 8 µL
11. Place column into a 1.5 mL tube and centrifuge for 30-60 seconds to elute DNA
12. DNA is now ready for use or storage

DNA Clean - Zymogen Kit

Source:
https://files.zymoresearch.com/protocols/_D4003T_D4003_D4004_D4013_D4014_DNA_Clean_Concentrator_-5_ver_1_2_1_LKN-SW__1.pdf

Materials

- DNA clean and concentrator-5 kit (Zymo)
- DNA samples
- 1.5 mL microcentrifuge tubes

Procedure

1. Add 2-7 volumes of DNA binding buffer to each DNA sample in 1.5 mL microcentrifuge tubes. Refer to table. Briefly vortex.

Table 1: DNA binding buffer to sample ratios for different applications

Application	DNA binding buffer: sample
Plasmid, genomic DNA (>2 kb)	2 : 1
PCR product, DNA fragment	5 : 1
ssDNA (e.g. cDNA, M13 phage)	7 : 1

2. Centrifuge at max speed for 30 sec and discard flowthrough
3. Add 200 µL DNA wash buffer, and centrifuge for 30 sec
4. Repeat wash step (step 3)
5. Run a dry spin to remove excess ethanol
6. Add > 6 µL DNA elution buffer directly to the column, and let sit for 1 min at room temperature
7. Transfer column to 1.5 mL microcentrifuge tube and centrifuge for 30 sec
8. Check concentrations using NanoDrop spectrophotometer

DNA Clean - Qiagen Kit

Materials

- Qiagen Kit
    - Buffer PB
    - Buffer PE
    - Buffer EB
- Centrifuge
- DNA sample
- 1.5 mL tubes

Procedure

1. Mix 125 µL buffer PB with ~25 µL of DNA sample
2. Centrifuge for 1 min at 17,900 x *g*
3. Add 750 µL buffer PE and centrifuge for 1 min
4. Dry spin for 1.5 min
5. Transfer column to 1.5 mL tube and add 50 µL buffer EB, let sit for 3 min
6. Centrifuge for 1 min

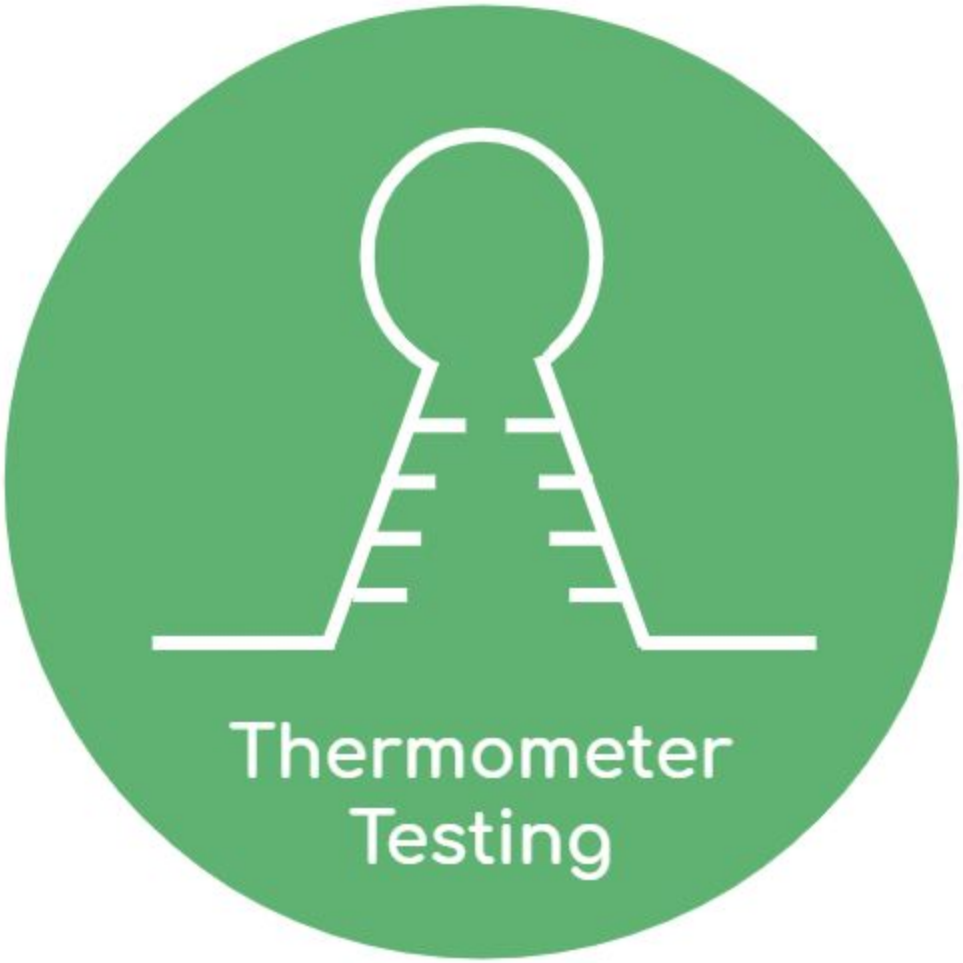
DNA Sequencing

Materials

- Universal sequencing primer
- DNA plasmid
- Water

Procedure

1. Followed protocols directly from Genewiz
    a. https://www.genewiz.com/en/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines/Sample-Preparation
2. We prefer to premix primers with minipreps DNA, which simply involved mixing water, DNA and primers until the final concentration is in accordance with the specified guidelines


Thermometer
Testing

RNA Thermometer Testing in Solid Culture using Spot Inoculation

Materials

- Plate with antibiotic
- Saturated culture
- Macroscope

Procedure

1. Spot 1 uL of culture for each construct onto plate
2. Let grow for over 10 hours
3. Analyze using macroscope

RNA Thermometer Fluorescence at End Points

Materials

- Media
- antibiotic
- inducer
- saturated strains of bacteria
- 96 well block
- serological pipette
- 25 ˚C incubator
- 30 ˚C incubator
- 37 ˚C incubator
- plate reader

Procedure

4. Prepare a stock of media with antibiotic and another stock with antibiotic and inducer
5. Using a serological pipette, fill 96 well blocks with 900 µL of appropriate media stock
6. Inoculate each well with the appropriate strain of bacteria
7. Repeat 2 times for a total of 3 blocks
8. Incubate one block at 25˚C, 30˚C and 37˚C for 8 hours
9. Measure using a Plate reader and Spark analysis tool

RNA Thermometer Fluorescence Time Course Measurements

Materials

- 96 well block
- Plate reader
- IPTG
- LB liquid medium
- Strains containing plasmids
- shaking incubator
- sulfur rhodamine

Procedure

Bacterial Strain Preparation

1. Grow in 2 mL LB liquid medium at 37 ˚C until saturation for about 8 hours
    a. OD600 = 0.1
2. From the 8 hour culture of 2 mL LB, freeze 1 mL of the saturated culture for glycerol stocks for further testing
3. From overnight culture (8 hr growth), measure OD in a 1:30 dilution,
    - To measure OD in 1:30 dilution, add 23.33µL of culture to 676.67µL LB (final 700 µL)
4. dilute to 0.01, then grow to OD600 = 0.1 in LB in a 37 ˚C shaking incubator at 250 rpm. This takes approximately 1.5 hours.

Plate Preparation

1. Add 100µL of 2mM of IPTG to each well
2. add sulfur rhodamine for normalization
    a. we recommend starting at 10mg/L and then 2x dilute from there
3. Add 100µL of culture to each well.
4. Grow,in plate reader at targeted temperature

RNA Thermometer Fluorescence Time Course with Ramping Temperatures

Materials

- 96 well block
- Plate reader
- IPTG
- LB liquid medium
- Strains containing plasmids
- shaking incubator
- sulfur rhodamine

Procedure

1. Prepare a stock of media with antibiotic and another stock with antibiotic and inducer
2. Using a serological pipette, fill 96 well blocks with 900µL of appropriate media stock
3. Inoculate each well with the appropriate strain of bacteria
4. Repeat 2 times for a total of 3 blocks
5. Measure using a Plate reader and Spark analysis tool with a protocol that spends ~5 hours at 25°C, ~4 hours at 30°C and ~2 hours 37°C
    a. these times should be calculated by looking at the growth curves of the particular strain you are using and ensuring that each temperature the OD will increase by approximately the same amount


Plant Work

Plant Nutrient (PN) Medium

Materials

- 1 L bottles
- Autoclave tape
- Bacto Agar
- beakers
- Components in Table 1

Procedure

1. Prepare 1 L bottle by labeling with "PN," the date, and initials
2. Place a piece of autoclave table on each cap
3. Remove Ferric EDTA from -20 ˚C freezer so it may thaw while you are preparing the bottles
4. Weigh out 3.7 g Bacto Agar for each 1 L bottle
    a. Liquid PN may be made leaving out Bacto Agar
5. Fill three beakers with the components in table 1
6. Mix the media well
7. Distribute 600 mL PN medium in each 1 L bottle (final Bacto Agar concentration of 0.6%)
8. Close caps loosely
9. Submerge in sterilization monitor in center bottle of each autoclave tray
10. Autoclave 45 minutes on liquid cycle
11. Swirl bottles and tighten caps before placing autoclaved PN on the shelves

Table 1: Components for Step 5

Component	Volume	Concentration in 1X PN
milliQ water	2937 mL	-
250 mM KPO_4 (pH 5.5)	30 mL	2.5 mM
1 M KNO_3	15 mL	5 mM
1 M $MgSO_4$	6 mL	2 mM
1 M $Ca(NO_3)_2$	6 mL	2 mM
Ferric EDTA (1000X)	3 mL	52 µM
Micronutrients (1000X)	3 mL	

PN Plate Preparation

Materials

- Desired PN concentration stock (0.6% or 1%)
- Any supplements (sucrose, glucose, IAA, trehalose, arabinose, etc) for plant media
- Plates
- BSC

Procedure

1. Take a stock bottle of your desired concentration of PN. Heat it until the PN is entirely melted. (Place in biosafety cabinet)
2. Cool media to 55 ˚C
3. Add supplements of desired concentration. Swirl to mix
4. Open package of plates in biosafety cabinet, and label.
5. Pour media into plates, fill to ½-⅓ of plate depth
6. Cool media until solidified
7. Place in plates original packaging bag, seal with tape
8. Store in 4 ˚C fridge until ready for use.
    a. For plates with IBA/IAA they should be wrapped in foil for storage to prevent light degradation)

Plant Seed Sterilization

Materials

- Eppendorf tubes
- Plant seeds
- Bleach solution (% NaOCl and 0.01% Triton X-100)
- MQ water
- agar

Procedure

1. Place seeds in Eppendorf tube
2. Add bleach solution (3% NaOCl and 0.01% Triton X-100) to the tube (~200-800 uL depending on the amount of seeds)
    a. Bleach solution is made with:
        i. 25 mL 6% NaOCl (ensure it is simple bleach, scented/enhanced bleaches kill seeds)
        ii. 25 mL water
        iii. 50 uL 10% Triton X-100
    b. Bleach solution degrades under light, so keep bottle wrapped in foil
3. Vortex tube to break up clumps of seeds
4. Allow tubes to sit at room temperature for 10 minutes, vortex occasionally
5. Aspirate off the bleach, taking care not to suck up the seeds along with it (you may leave a little bleach)
6. Wash the seeds 2X with sterile MQ water
7. Add 1 mL water, allow seeds to settle, then aspirate off the water
8. Resuspend in 0.1% agar (~500-1,000 uL depending on the amount of seeds)
9. Stratify to help even out germination times
    a. Place in 4 ˚C fridge for 1-5 days

Seed Plating

Materials

- Eppendorf tubes with sterilized and stratified seeds
- Plant plates
- Micropore gas permeable tape
- incubator

Procedure

1. Remove Eppendorf tube with stratified seeds from 4 ˚C fridge
2. Use a micropipette to uptake the seeds
3. Remove pipette tip from micropipette
4. Hold the tip horizontally
5. Use your thumb as a manual pipette by placing it over the large opening of the tip and pressing when you want to remove liquid
6. Hold uncovered, labeled plate vertically with one hand. With the other hand, tap the small opening of the pipette to the media. This will create a vacuum that will remove small amounts of the liquid, taking seeds along with it
7. When plating for vertical growth, place seeds in a single line toward the edge of the plate you will be using as the top of the plate
8. When plating for horizontal growth, seeds may be placed anywhere along the plate
9. Lid the plate and seal with micropore gas permeable tape (surgical tape)
10. Incubate seeds in the plant growth chambers using yellow light
    a. Incubator conditions
        i. 22 ˚C and 24 hour light

Inoculation of *A. thaliana* with *P. putida*

Materials

- Plant items
    - *A. Thaliana seeds*
    - PN glucose media
    - fsquare petri dishes
    - tape to wrap plates
    - plant incubator
    - inducer
- Bacteria items
    - *P. putida*
    - LB media
    - Antibiotic
    - bacterial incubator

Procedure

Seed preparation

1. Sterilize *Arabidopsis Thaliana* Col-e seeds for 24 hours

Plate preparation

1. Pour 40 mL of PN 0.01% glucose with appropriate concentration of inducer into square fisher petri dishes

Bacterial preparation

1. Grow p. putida for > 8hr in 3 mL of LB with appropriate antibiotic at 30˚C
2. Spin down bacterial culture in 2600 x *g* for 15 min
3. Resuspended in 2 mL sterile DI water
4. Dilute to an OD of 0.2 (~10^8 CFU/mL)

Spot inoculation

Note: Must be done in a biosafety cabinet

1. Using a p1000 tip, seed 5-7 seeds per plate
2. Using a p20 micropipette, drop 10-15µL of the bacterial culture directly onto of the seeds
3. Let dry for at least an hour before moving
4. Tape plates together starting at the side opposite from the seeds. Wrap around so that the bottom is covered twice. Then stand vertically and tape two plates together
5. Incubate in a humidity controlled, constant light, plant incubator at 22˚C

Ampicillin and Carbenicillin Experiments with *P. putida*

Materials

- 5 mg/mL stock of carbenicillin
- 1 mg/mL stock of ampicillin

Procedure

1. Aliquot 2 mL of LB30 into 10 wells in 24 well block
2. From a 5 mg/mL stock of carbenicillin, add 20µL to first well, 18µL to second well, and so on to 2 µL in 10 well
3. Pick a colony from *P. putida* pSPB874 and add to each well
4. Put in 30 ˚C incubator