Appendix 1

Full lab protocol for OIL PCR

OIL-PCR protocol

*Please email Peter Diebold, pd378@cornell.edu, for questions regarding this protocol

General workflow

1. Design and test primers
2. Nycodenz purify cells from sample (day 1)
3. Quantify cell concentration of purified cells (day 1)
4. Perform OIL-PCR (day 2)
5. Break the emulsion and recover the aqueous phase (day 2)
6. Bead purify the fusion PCR products from the aqueous phase (day 2)
7. Perform nested qPCR to choose cycle number for each sample/primer combination (day 3)
8. Perform the nested PCR with cycle number from qPCR (day 3)
9. Bead purify the DNA (day 3)
10. Perform the Illumina Indexing PCR (day 4)
11. Bead Purify the DNA (day 4)
12. Quantify the DNA concentration (day 4/5)
13. Pool reactions for Illumina sequencing (day 5)

Design primers

1. Collect sequences for the desired target. For ARGs, I pulled all the available sequence from the CARD database.
   a. In many cases the diversity of the genes is too great to design a single primer set. In this case I looked through the genes on CARD and annotated the ones that were most prevalent or medically relevant and tried to design primers for them.
   b. Sometimes more than one primer set will be needed to target a diverse group of genes.
2. Identify potential priming regions
   a. First, I mark regions with GC content between 40 and 60%. Snapgene has a function to display GC content as a graph.
   b. I also align the sequence variants and mark regions that are highly similar for priming.
   c. It is also advantageous to design primers which span a region of dissimilarity for detecting gene variants when possible.
3. Design multiple potential primers with the following parameters
   a. GC content between 40 and 60%
   b. Tm in snapgene of approximately 58 (This is the number I've always used)
   c. I try to avoid too many degenerate bases, but they are often unavoidable in which case I try to keep degeneracies away from the 3' end
   d. Design multiple primers without worrying about fragment size immediately
   e. When designing highly specific primers (i.e. species targeting), the NCBI primer blast is a useful tool
4. Search for potential combinations of the primers which will work for OIL
   a. The fusion primer and Round one primer can be far apart, although I try to keep it short if possible
   b. The nested primer and the fusion primer fragment should not be more than 200 bp including the primers
   c. I will often design primers so that I can try them in multiple combinations to see which work best together.
5. Add the fusion primer and nested primer tails to the 5' end
   a. Fusion tail: GWATTACCGCGGCKGCT
   b. Nested tail: ACACGACGCTCTTCCGATCT
6. Test the primers:
   a. Primers should be tested in a mock OIL-PCR mastermix

i. It is the same as the normal master mix, but without lysozyme and using the manufacturers recommended concentration of Phusion polymerase. I usually do 20–25 µl reactions

b. I like to do SYBR based qPCR for the nested PCR, but it's not necessary. Just make sure something amplifies. Simply add 1× SYBR and optionally 1× ROX to the master mix

c. I also will sequence the final fusion constructs

Nycodenz purify cells

Reagents/equipment

- Stool stored in PBS + 20% glycerol + 0.1% L-cysteine
- Cold PBS
- Cold PBS + 20% glycerol + 0.1% L-cysteine
- Cold 80% Nycodenz (VWR 100356–726)
- 2 ml microcentrifuge tubes
- 40 µm nylon mesh screen (Falcon 352235)
- Cryogenic vials
- Dry Ice/EtOH slurry or liquid N2
- Vortexer
- Refrigerated centrifuge with a swing bucket rotor cooled to 4˚C
- 1 ml filter pipette tips with approximately 1 cm cut from the end for pipetting stool (wide bore tips to not have a large enough orifice)

Protocol: Perform all steps on ice and in a refrigerated centrifuge at 4˚C

1. Vortex stool sample to thoroughly homogenize
2. Dilute approximately 800 µl of stool 1:1 in cold PBS to reduce sample viscosity (dilute further for particularly viscous samples). Use the cut pipette tips to transfer stool.
   - Note: too little stool results in a thin stool pellet which makes removing the cells more difficult; however, too much stool/glycerol can interfere with the density gradient. Performing a run with 'practice' stool is recommended.
3. Add 300 µl of 80% Nycodenz solution to the bottom of a 2 ml microcentrifuge tube. Be sure to pipette the solution directly to the bottom of the tube
4. Overlay 1.6 ml of stool slurry on top of the Nycodenz. Be gentle so that the stool does not mix with the Nycodenz.
5. Centrifuge the tubes for 40 min at 10,000 G in a swing bucket rotor
6. Remove the upper phase and discard
7. Add 500 µl of cold PBS and pipette up and down to wash the lighter colored cell layer from the stool pellet. Sometimes there is a lighter and darker layer of cells with the darker being harder to suspend. Check on a scope to confirm relatively pure cell fractions.
8. Pass cells through the 40 µm nylon mesh screen
9. Optional: Filter cells through a 5–8 µm filter to remove cell clumps and clean the cells further. Expect significant loss of cells in the filter
10. Dilute cells 1:1 in PBS + 20% glycerol + 0.1% L-cysteine. Set some aside for cell quantification
11. Freeze multiple aliquots of the purified cells in cryogenic vials. Ideally flash freeze and store at −80˚C

Quantify cell concentration

Reagents/equipment

- Nycodenz-purified cell fraction
- 20 µm counting chamber (VWR 15170–048)
- Microscope with 40× phase contrast objective

Protocol

1. Dilute cells to a countable concentration. Too many cells or too few will make counting difficult. Usually 1:10 or 1:100 is good.
2. Pipette 10 µl of cells on the counting chamber and add the coverslip
3. Place the slide on the microscope and capture images of at least five squares
    - Note: The scope cannot perfectly focus on all planes within the depth of the counting chamber. You must focus somewhere in the middle so that most cells are blurry but visible for counting
4. Use FIJI to count cells in each square and calculate the final concentration based on the counting chamber volume and dilution factor
    - Because of the imperfect focus, the counting must be done by hand using the 'cell-counter' plugin

Note: Fluorescent staining and imaging the cells could improve counting accuracy, but any stray stool particles will fluoresce strongly. Also, a flow cytometer could be used if available.

OIL-PCR

Reagents/equipment

- Thawed Nycodenz-Purified Cells
- Cold 1× PBS
- Cold BioRad Emulsion Oil (BioRad 1863005)
- dsDNase (Thermo Fisher EN0771)
- PCR Reagents:
    - 5X DF Buffer (Thermo Fisher F520L)
    - dNTPs (NEB N0447L)
    - 100 µM 16S reverse primer AP27 (TTTTTTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT)
    - 100 µM forward primer
    - 10 µM fusion primer (5' tail GWATTACCGCGGCKGCT)
        - Multiplexed reactions will have up to 3 forward and three fusion primers
    - $MgCl_2$ (NEB M0535L)
    - 100 mM Ammonium Sulfate
    - 100 mM DTT
    - BSA (NEB B9000S)
    - Ready-Lyse Lysozyme (Lucigen R1810M)
    - Phusion Hot Start Flex DNA Polymerase (NEB M0535L)
- 96-Well PCR plates (Eppendorf 0030 128.648)
    - Do not swap plates as it has been reported that the different plates can affect emulsion stability
- Retch Mixer Mill MM 400 with plate adapters (Qiagen/MoBio #11990).
    - Also use adapter 1193 for tube-based format
    - Any bead beater would be a suitable alternative to the Retch.
    - A vortexer could also be used however we have not verified the parameters

Equipment for automated protocol

- 500 µl Deep-Well Plate (Eppendorf 00.0 501.101)
- Thick Foil Seal (Axygen PCR-AS-600)
- 8-Well PCR Strip Tube
- Disposable reservoir for multichannel pipettes
- 10 µl multichannel pipette
- 200 or 300 µl multichannel pipette
- Eppendorf EP Motion setup to run 'OIL_PCR-A' program

Protocol

1. Standardize cell stock to 10^5 cells/µl in cold PBS using the calculated concentration
   - Standardize directly to 10^4 cell/µl if stocks are dilute
2. Add 1 µl of Ready-Lyse Lysozyme to each tube of cells and incubate for 10 mins at RT. Return cells to ice when complete
3. Prepare the PCR MasterMix:
   - 50 µl reactions for the manual tube-based format
   - When multiplexing, there will be up to three forward and three fusion primers total. Adjust the master mix to account for the extra primers
4. Aliquot 96 µl of OIL mastermix into each well of a 500 µl DWP on ice
   - Or 48 µl into a 1.5 ml tube for the tube-based method
5. Dilute the dsDNase treated cells 1:10 to a final concentration of 10^4 cell/µl in an eight-well PCR strip tube for multichannel pipetting
   - This dilution step reduces the final concentration of dsDNase in the OIL reaction to prevent degradation
6. Using the 10 µl multichannel, transfer 4 µl of cells to OIL-PCR mastermix. Gently pipette to mix
   - Add the cells directly to the bottom of each well. Avoid getting any on the side of the plate
   - It is extremely important to keep the cells cold and work fast to prevent premature lysis of cells before emulsification
   - Add 2 µl of cells individually when performing the tube-based method
7. Seal the plate and vortex to mix
8. Quick spin the plate to return all liquid to the bottom of the plate
9. Carefully vortex the reactions a second time for 30 s. Try to keep the liquid at the bottom of the wells. A high-speed plate shaker is best if available
   - Note: Mixing the cells evenly through the master mix is extremely important. Unmixed droplets of cells on the side of the tube will result in poor isolation of cells.
10. Quickly add 200 µl of emulsion oil to each well using a multichannel pipette
    - 300 µl for the tube-based method
11. Seal the plate with a foil seal and shake for 20 s at 30 Hz
    - 25 Hz for 30 s in the tube based. Flipping is unnecessary if the tubes are in the center
12. Flip the plate so the inside arc is now on the outside and shake for another 20 s
13. After emulsification the reaction can be kept at room temperature to for Lysis to begin
14. Run the OIL_PCR-A program to consistently aliquot the emulsion to PCR plate
    - This step can be done by hand (70 µl mix to four wells of the plate), but it is difficult to evenly distribute the emulsion between wells. The robot is used to properly mix the emulsion before each transfer
    - Perform the transfer by hand with the tube-based method
15. Seal the plates and run the OIL-PCR thermocycle program:
    - Note 1: The program incubates at 30˚C and not 37˚C. This was implemented because of concern that the dsDNase or endogenous nucleases could degrade DNA too quickly at higher temperatures. 37 would likely work better for lysis but the method has not been changed for consistency
    - Note 2: Slow temperature ramp rates were used to allow even heating through the emulsion
    - Note 3: The emulsion will separate to the top of the reaction and congeal which is normal

Reagent	Stock concetration	Final concentration	Volume (µl)
H_2O			to 100 µl
DF Buffer	5×	1×	20
dNTPs	10 mM	250 M	2.5

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Reagent	Stock concetration	Final concentration	Volume (µl)
16 S-R AP27	100 µM	2 µM	2
pForward	100 µM	1 µM	1–3
pfuse	10 µM	0.01 µM	0.1–0.3
$MgCl_2$	50 mM	1.5 mM	3
AmSulfate	100 mM	5 mM	5
DTT	100 mM	5 mM	5
BSA	20 mg/ml	4 mg/ml	20
Lysozyme full	variable	300 U/µl	0.792
Polymerase	2000 U/ml	100 U/µl	5
template	10^4 cells/µl	400 cell/µl	4
Total			100

		5:00	30
		10:00	95
38×		0:05	95
		0:30	54
		0:30	72
		2:00	72
		hold	4

Breaking the emulsion

Reagents/equipment

- Perfluorooctanol (Krackeler Scientific 45-370533-25G)
- TE

For automated version:

- Centrifuge capable of spinning deep-well plates
- 300 µl filter pipette tips for the robot
- 300 µl multichannel tool
- 30 ml reservoirs

Automated version

1. Open the robot protocol for 'OIL_PCR_B' or 'OIL_PCR_B_Ampure'
   a. The ampure version transitions directly to the bead cleanup after breaking the emulsion
2. Setup the stage as shown in the program
3. Vortex the plate to break up the emulsion before carefully opening
4. The robot will first pool the reactions into a 500 µl 96-well deep-well plate
5. The robot will then add 60 µl TE and 70 µl perfluoro-1-octanol
6. Seal the plate with foil and place on the shaker at 30 Hz for 20 s per side
7. Spin down the plate 5000G for 1 min
8. Return the plate to the robot and it will remove the lower oil phase and discard it in the waste reservoir
9. Then it will pipette off the upper aqueous phase into a 96-well PCR plate, or the other half of the DWP if using the ampure version

a. If doing the ampure version, it will continue as describe in the AMPure XP automated protocol

Manual version

1. Vortex the plate to break up the emulsion before carefully removing the foil seal without cross contaminating wells
2. Pool the four reactions into a 1.5 ml tube, being sure to mix well between pipette steps to capture as much of the emulsion as possible
3. Centrifuge at 500G for 1 min and remove the lower oil phase
4. Add 50 µl TE and 70 µl Perfluorooctanol
5. Vortex at max speed for 30 s
6. Centrifuge at 500G for 1 min
7. Carefully remove the upper phase and transfer to a PCR strip for Ampure XP bead purification

Ampure XP cleanup

Reagents

- AMPure XP beads (Beckman A63880)
  - 96-Well plate magnetic separator (Eppendorf Magnum FLX)
- Any magnet will work for the manual protocol
- TE
- 70% EtOH

Manual specific:

- Multichannel reservoir
- 100, 200, or 300 µl multichannel pipette

Automation specific:

- 300 µl and 1000 µl filter tips
- 300 and 1000 µl multichannel tools
- 30 and 100 ml reservoirs
- 500 µl deep-well plate (Eppendorf 00.0 501.101)
- 96-Well PCR plate for elution (Eppendorf 0030 128.648)

Manual protocol

This can be done in either a full 96-well plate or also individual 8-well strip tubes. If using strip tubes, you will need to fashion some kind of adapter to hold them upright in the magnet. The top of some 200 µl tip boxes often works well.

1. Add a ratio of 0.8x beads to each reaction (e.g., 80 µl beads for 100 µl PCR)
   a. It's better to have too much than too little
2. Pipette or vortex to mix and allow 5 min for the DNA to bind the beads
3. Perform a brief spin to return all liquid to the bottom of the wells
4. Place the tubes on the magnet for 2 min to pull down the beads
5. Use a multichannel to remove the supernatant
6. Use a multichannel to add 100 µl of EtOH to each well. Pipette up and down to wash without disturbing the beads and immediately remove and discard the supernatant
7. Repeat step six for a second wash
8. Remove from the magnet and dry at RT for 10 min
9. Add the desired amount of TE to each well (I usually default to 25 or 50 µl)
10. Mix well either by pipetting or vortexing
11. Allow five mins for the DNA to fully elute
12. Place on the magnet and allow 2 min for the pull-down
13. Transfer the supernatant to a fresh plate/strip-tubes with a multichannel pipette

Automated protocol

1. Setup the robot as described in the 'Ampure Cleanup' Protocol
   a. Fill two 30 ml reservoirs with appropriate volumes of Beads and TE
   b. Fill a 100 ml reservoir with EtOH
   c. Place the tips, reservoirs, waste, magnet, and plates as shown in the program
2. Adjust the Ampure XP transfer volume to be 0.8× of the PCR volume
3. Adjust the TE volume for the desired elution
4. Begin the program. It performs all the same steps as the manual one.

Run nested qPCR

Reagents

- Luna universal qPCR master mix (NEB M3003L)
- Nested Target Primers
- Reverse 16S Primer AP28

Protocol

For multiplexed reactions, there will be an individual qPCR assay for each of the genes. DO NOT MULTIPLEX THE NESTED PCR REACTIONS

1. Make the qPCR master mix with the following recipe

Reagent	Stock concetration	Final concentration	Volume (µl)
H_2O			to 20
Luna Buffer	2×	1×	10
Nest Primer	100 uM	300 nM	0.06
16 S-R AP28	100 uM	300 nM	0.06
Template			2–5

2.Aliquot the master mix into a qPCR plate and use a multichannel pipette to add template to the reaction

3.Run the reactions with the following program

	2:00	95
38×	0:15	95
	0:15	55
	0:20	68
	1:00	65
	0.15˚C /s	95

4. Check melt curves to confirm clean amplification
5. Select cycle numbers for each sample equal to the Ct value ± two cycles

Run nested PCR

Reagents

- 5X HF Buffer (NEB M0535L)
- dNTPs (NEB N0447L)
- 100 µM 16S reverse primer AP28 (GAGTTCAGACGTGTGCTCTTCCGATCTGGACTAC)

- 100 µM Nested primer (5' Tail ACACGACGCTCTTCCGATCT)
- 100 µM Blocking F (TTTTTTTTTTCAGCMGCCGCGGTAATWC/3SpC3/)
- 100 µM Blocking R (TTTTTTTTTTGWATTACCGCGGCKGCTG/3SpC3/)
- Phusion Hot Start Flex DNA Polymerase (NEB M0535L)
- OIL-PCR Template

Protocol

For multiplexed reactions, there will be an individual reaction for each of the genes. DO NOT MULTIPLEX THE NESTED PCR REACTIONS!

1. Prepare enough mastermix without template for all samples as follows:

Reagent	Stock concetration	Final concentration	Volume (µl)
H2O			to 100 µl
HF Buffer	5×	1×	6
dNTPs	10 mM	200 µM	0.6
Nest Primer	100 µM	30 nM	0.09
16 S-R AP28	100 µM	30 nM	0.09
Block F	100 µM	3.2 µM	0.96
Block R	500 µM	3.2 µM	0.96
Polymerase	2000 U/µl	20 U/µl	0.3
template			2–5
Total			30

2. Aliquot master mix minus template volume to wells of a 96-well plate
3. Add Purified template with a multichannel pipette
4. Mix the reactions and transfer 15 µl of each reaction to a fresh plate for replicates (2 × 15)
5. Run the reactions as follows:

	2:00	98
Variable	0:05	98
	0:30	55
	0:30	72
	5:00	72

6. After cycling, pool the replicates and perform the Bead cleanup

a. Thermolabile Exonuclease I (NEB M0568S) could be used instead of a bead cleanup at this step to save time and reagents. The exonuclease will degrade the primers from the nested reaction and then is head inactivated.

Run index PCR

Reagents

- 5X HF Buffer (NEB M0535L)
- dNTPs (NEB N0447L)
- 5 µM Forward Index
- 5 µM Reverse Index
  - Alternatively, a plate of premixed primers can save time in the long run. In other words, prepare a 96-well plate of primers, where each well has a unique combination of index primers
  - Primer sequences are in the ***Supplementary file 2***
- Phusion Hot Start Flex DNA Polymerase (NEB M0535L)
- Nested PCR Template

Prepare enough mastermix for all samples, without template or primer as follows:

Reagent	Stock concetration	Final concentration	Volume (µl)
H_2O			to 25 µl
HF Buffer	5×	1×	5
dNTPs	10 mM	200 µM	0.5
F Index	5 µM	100 nM	0.5
R Index	5 µM	100 nM	0.5
Polymerase	2000 U/µl	20 U/µl	0.25
template			2–5
Total			25

8. Aliquot the master mix to a 96-well plate (minus template and primer volume)
9. Add index primers to the plate individually
10. Primers can be aliquoted into PCR strip tubes for multichannel pipetting across the plate
a. dilution of primers to 1 uM can make pipetting easier
b. Use a multichannel to transfer purified, nested PCR template to each well
11. Run the following program for indexing:
12. After amplification, perform a bead purification of the reactions

	1:00	98
20 cycles	0:15	98
	0:30	56
	0:45	72
	2:00	72

Quantify the DNA concentration

Use the QUANT-IT pico green dsDNA assay kit (Invitrogen P7589) as described in the manufactures instructions to quantify the concentration of DNA and measure using a fluorometric plate reader.

Pool the reactions

Reactions were pooled to a standard concentration from all reactions and submitted for sequencing.