DropSynth 2.0 emulsion synthesis protocol (Aug 2019)

1. Prepare the OLS pool
    - Make a 1/10 dilution of the OLS chip pool.
    - Prepare mixtures of forward and reverse subpool amplification primers for each subpool, with 10 µM final concentration of each primer.
2. Amplify subpools.
    - For each subpool, run a qPCR to determine the number of cycles required for amplification. Amplifications are stopped several cycles before plateauing to prevent over-amplification of the libraries.
    - Amplify each subpool using NEB Q5.
        - 1 µL template (1/10 OLS pool dilution)
        - 1.25 µL subpool specific primer 10 µM ampF
        - 1.25 µL subpool specific primer 10 µM ampR
        - 21.5 µL UltraPure Distilled Water (Invitrogen)
        - 25 µL NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs)
        - TOTAL: 50 µL
        - PCR protocol:
            1. 45 sec 98ºC initial denaturation
            2. 15 sec 98ºC denaturation
            3. 30 sec 58ºC annealing
            4. 15 sec 72ºC extension
            5. Go to step 2, repeat based on the number of cycles determined by qPCR.
            6. 1 min 72ºC final extension
    - Column purify amplified oligos using a DNA Clean & Concentrator -5 (Zymo Research).
    - Run PCR products on gel. Look for higher MW products, indicative of overamplification. Excessive low MW products may indicate chip synthesis issues.
    - Size select, using gel extraction, if necessary.
    - Create 20 pg/µL dilutions of each amplified subpool.
3. Bulk amplify subpools.
    - Run a second PCR using a biotinylated FWD amplification primer, with sufficient tubes to make 5 ug to 10 ug of PCR product.
        - 1 µL of 20 pg/µL subpool dilution
        - 1.25 µL subpool specific primer mix 10 µM biotinylated ampF
        - 1.25 µL subpool specific primer mix 10 µM biotinylated ampR
        - 21.5 µL UltraPure Distilled Water (Invitrogen)
        - 25 µL NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs)

        - TOTAL: 50 µL
        - PCR protocol:
            1. 45 sec 98ºC initial denaturation
            2. 15 sec 98ºC denaturation
            3. 30 sec 58ºC annealing
            4. 15 sec 72ºC extension
            5. Go to step 2, 18X
            6. 1 min 72ºC final extension
    - Pool and column purify using a DNA Clean & Concentrator -25 (Zymo Research).
4. Nicking.
    - Nick the bulk amplified subpools. Split the following across multiple tubes depending on the amount of DNA to be processed. In each 1.5 mL tube add:
        - 15 µL Nt.BspQI (10U/µL) (New England Biolabs)
        - 5 to 10 ug of DNA
        - 15 µL NEBuffer3.1 (New England Biolabs)
        - UltraPure Distilled Water (Invitrogen) to 150 µL total
    - Leave at 50ºC overnight with shaking >1500 RPM.
5. Capture and remove the short biotinylated fragment.
    - Wash 50 µL streptavidin M270 Dynabeads (Invitrogen) for each 1.5 mL tube in the nicking reaction, as per manufacturer's instructions and resuspend in 2X B&W buffer.
    - Add 50 µL of washed beads to the 150 µL nicking reaction in each tube.
    - Incubate at 55ºC with 800 RPM shaking for at least 1 hour.
    - Move all 1.5 mL tubes to a 55ºC water bath.
    - Place the tube so that solution is just below the surface of the water. Hold a strong magnet underwater against the side of the tube to magnetically separate Dynabeads. Pipette the supernatant, which contains the processed oligos and save them in a new container. Remove the tube with the Dynabeads from the magnet.
    - Add 100 µL of UltraPure Distilled Water (Invitrogen) to the tube and resuspend the beads. Incubate these at 55ºC for another 30 min and then repeat the procedure to recover the supernatant again while leaving the Dynabeads behind.
    - Repeat this procedure for all tubes as necessary.
    - Pool processed oligos (supernatant) for each subpool and column purify using a DNA Clean & Concentrator -5 (Zymo Research).
6. Capture processed oligos with barcoded beads.
    - Take 20 µL of the pooled barcoded beads. These are in stored in 2X B&W buffer (high ionic concentration) which may interfere with ligation reaction. Resuspend them in 20 µL UltraPure Distilled Water (Invitrogen).
    - Mix the processed DNA with the barcoded beads:
        - 1.3 ug processed DNA (~12 pmol)
        - 20 µL pooled barcoded beads (~6 million beads, binding capacity 1.3 ug DNA)

        - 10 µL 10X Taq ligase buffer (New England Biolabs)
        - 4 µL Taq ligase (40 U/µL) (New England Biolabs)
        - UltraPure Distilled Water (Invitrogen) to 100 µL
    - Overnight cycling (>2 hr incubation at each of the following temperatures) (13 hr), use shaking to prevent beads from settling down:
        - 3 hours @ 50ºC
        - Ramp to 40ºC for 3h, 0.1ºC/sec
        - Ramp to 30ºC for 3h, 0.1ºC/sec
        - Ramp to 20ºC for 2h, 0.1ºC/sec
        - Ramp to 10ºC for 2h, 0.1ºC/sec
    - Wash 3 times at 4ºC using 2X B&W buffer. This is important for removing unbound oligos in order to increase specificity.
    - Wash twice at RT using 2X B&W buffer
    - Re-suspend in 100 µL Elution Buffer (Qiagen) (~60k beads/µL)
7. Emulsion assembly (ePCA).
    - Setup emulsion. All of this procedure should be done on ice. FWD and REV assembly primers contain ITR overhangs which will be used for single-primer suppression PCR. Add BtsI-v2 only at the very last step. Try to minimize the time between adding the BtsI-v2 and vortexing the emulsion.
        - 40 µL of loaded beads (~500 ng DNA)
        - 0.5 µL 100 µM AsmF_40bpITR
        - 0.5 µL 100 µM AsmR_40bpITR
        - 50 µL KAPA HiFi 2X Mastermix (KAPA Biosystems)
        - 1 µL BSA (New England Biolabs)
        - 1 µL UltraPure Distilled Water (Invitrogen)
        - 7 µL BtsI-v2 (New England Biolabs) (<u>add last</u>)
        - TOTAL: 100 µL
    - Mix at low speed in vortexer to resuspend beads.
    - Add 600 µL Droplet Generation Oil for EvaGreen (Bio-Rad) to a 1.5mL non-stick tube.
    - Add 100 µL aqueous phase to the bottom of the oil phase.
    - Vortex at Max Speed in foam holder taped down for 3 minutes. If doing multiple emulsions, do this one at a time. We use a Vortex Genie 2 (Scientific Industries) at max speed.
    - After vortexing all emulsions, place each emulsion into PCR tubes with 100 µL in each tube. Use a P1000 tip to avoid disturbing the emulsion. Most of the droplets will float to the top of the tube, try to get as much of this as possible and distribute this over multiple PCR tubes.
    - PCR Cycling
        - 55ºC for 90 min (allow BtsI-v2 to cleave DNA from the beads)
        - 94ºC for 2 min (initial denaturing)
        - 94ºC for 15 sec (denaturing)
        - 57ºC for 20 sec (annealing)

        - 72ºC for 45 sec (extension)
        - Go to step 3 for additional 60 cycles
        - 72ºC for 5min (final extension)
        - 4ºC forever
8. Break the emulsion:
    - After ePCA, pipet out the entire volume of droplets from each PCR tube into a 1.5 mL tube.
    - Add 100 µL of 1*H*,1*H*,2*H*,2*H*-Perfluoro-1-octanol (Sigma Aldrich) for each 100 µL of PCR reaction combined in the 1.5mL tube.
    - Vortex at maximum speed for 1 min.
    - In a centrifuge, spin down at 15,500 x g for 10 min.
    - If droplets are still present, vortex and centrifuge again.
    - Remove upper aqueous phase by pipetting, avoiding the oil phase.
    - Transfer this to a clean 1.5mL tube (this is the DNA).
    - Column purify using a DNA Clean & Concentrator -5 (Zymo Research).
9. Size selection.
    - The amplicons will often be mixed with undesired lower-molecular weight assemblies. Removing these using size selection will increase final yield.
        - Gel extraction
            1. Run amplicons on a gel and extract the correct range and purify.
            2. Note: Typically there is not enough DNA after the ePCA to visualize on a gel, so this is often a blind extraction.
10. MutS treatment (optional)
    - Enzymatic error correction can be used to enrich for perfect assembly products. Here we use M2B2 magnetic beads (US Biological), which contain immobilized MutS and thus bind to and magnetically separate DNA containing mismatch-generated heteroduplexes.
    - Add 10 µL of M2B2 magnetic beads to size-selected assembly product.
    - Incubate at 20ºC with 1600 RPM shaking for at least 1 hour.
    - Immediately place on magnetic rack and extract supernatant.
    - Column clean the DNA using a DNA Clean & Concentrator -5 (Zymo Research).
11. Single-primer suppression PCR.
    - In this technique, self-annealing of inverted terminal repeats (ITRs) flanking the assembled genes competes with the annealing of a single primer which aligns to part of the ITR[3]. Shorter by-products tend to self-anneal, while correct assembly products anneal to the primer, resulting in proper amplification.
        - 1 µL template
        - 4 µL 10 µM suppression primer
        - 25 µL NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs)
        - UltraPure Distilled Water (Invitrogen) to 50 µL
        - PCR protocol:
            1. 3 min 95ºC initial denaturation

2. 15 sec 98ºC denaturation
3. 30 sec 58ºC annealing
4. 15 sec 72ºC extension
5. Go to step 2, determine cycles using qPCR.
6. 1 min 72ºC final extension

- Column purify using a DNA Clean & Concentrator -5 (Zymo Research).
- Check size distribution on gel or tapestation.
- Quantify DNA and proceed to downstream applications.