DropSynth emulsion synthesis protocol

The following protocol was used to assemble the PPAT library. All PCR steps were performed on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories).

1. Prepare the OLS pool
    - Make 1/5, 1/10, and 1/20 dilutions 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.
        - 1 uL template (test 1/5, 1/10, 1/20 OLS pool dilutions)
        - 1.25 uL subpool specific primer mix (10 uM FWD + 10 uM REV)
        - 22.75 uL UltraPure Distilled Water (Invitrogen)
        - 25 uL Kapa HiFi HotStart ReadyMix (2X) (KAPA Biosystems)
        - TOTAL: 50 uL
        - PCR protocol:
            1. 3 min 95ºC initial denaturation
            2. 45 sec 98ºC denaturation
            3. 15 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 Zymo 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/uL dilutions of each amplified subpool. (~91 million/uL)
3. Bulk amplify subpools.
    - Run a second PCR using a <u>biotinylated</u> FWD amplification primer, with sufficient tubes to make 4.5 ug to 9 ug of PCR product.
        - 1 uL of 20 pg/uL subpool dilution
        - 1.5 uL subpool specific primer mix (10 uM biotinylated FWD + 10 uM REV)
        - 22.5 uL UltraPure Distilled Water (Invitrogen)
        - 25 uL Kapa HiFi HotStart ReadyMix (2X) (KAPA Biosystems)
        - TOTAL: 50 uL
        - PCR protocol:
            1. 3 min 95ºC initial denaturation
            2. 20 sec 98ºC denaturation
            3. 15 sec 58ºC annealing
            4. 15 sec 72ºC extension
            5. Go to step 2, 18X
            6. 1 min 72ºC final extension
    - Pool and column purify PCR reactions using a Zymo Clean & Concentrator -5 (Zymo Research).

4. Nicking.
    - Nick the bulk amplified subpools. Split the following accross multiple tubes depending on the amount of DNA to be processed. In each 1.5 mL tube add:
        - 4.5 uL Nt.BspQI (10U/uL) (New England Biolabs)
        - 2 to 2.5 ug of DNA (final concentration ~16ng/uL)
        - 15 uL NEBuffer 3 (New England Biolabs)
        - UltraPure Distilled water (Invitrogen) to 150 uL
    - Leave at 50ºC overnight with shaking >1500 RPM.
5. Capture and remove the short biotinylated fragment.
    - Wash 50 uL Dynabeads M-270 Streptavidin (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 uL of washed beads to the 150 uL 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 uL 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 cleanup using a Zymo Clean & Concentrator -5 (Zymo Research).
6. Capture processed oligos with barcoded beads.
    - Take 18 uL of the pooled barcoded beads. These are in stored in B&W buffer (high ionic concentration) which may interfere with ligation reaction. Resuspend them in 18 uL 10 mM Tris-HCl buffered solution.
    - Mix the processed DNA with the barcoded beads:
        - 40 uL processed DNA (~1.3 ug,~12 pmol)
        - 18 uL pooled barcoded beads (~5 million beads, binding capacity 1.2 ug DNA)
        - 10 uL 10X Taq ligase buffer (New England Biolabs)
        - 4 uL Taq ligase (40 U/uL) (New England Biolabs)
        - 28 uL UltraPure Distilled water (Invitrogen)
        - TOTAL: 100uL
    - Overnight cycling (>2 hr incubation at each of the following temperatures) (13 hr), while shaking using an Eppendorf ThermoMixer C (Eppendorf):
        - 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 B&W buffer. This is important for removing unbound oligos in order to increase specificity.
    - Wash twice at RT using B&W buffer
    - Re-suspend in 100 uL Elution Buffer (Qiagen) (~50k beads/uL)
7. Emulsion assembly (ePCA).

- Setup emulsion. All of this procedure should be done in cold room on ice. Add BtsαI only at very last step. Try to minimize the time between adding the BtsαI and vortexing the emulsion.
    - 10 uL of loaded beads (~130 ng DNA)
    - 0.5 uL 100 uM FWD assembly primer
    - 0.5 uL 100 uM REV assembly primer
    - 50 uL Kapa2G Robust HotStart ReadyMix (2X) (KAPA Biosystems)
    - 1 uL BSA (New England Biolabs)
    - 31 uL UltraPure Distilled water (Invitrogen)
    - 7 ul BtsαI (New England Biolabs) (add last)
    - TOTAL: 100 uL
- Mix at low speed in vortexer to resuspend beads.
- Add 600uL Droplet Generation Oil for EvaGreen (Bio-Rad Laboratories) to a 1.5mL non-stick tube.
- Add 100uL aqueous phase to the bottom of the oil phase.
- Vortex at Max Speed in foam holder taped down for 3-4 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 uL 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 BtsαI 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. Adapted from pg 69 of the Bio-Rad Droplet Digital PCR Applications Guide:
    - After ePCA, pipet out the entire volume of droplets from each PCR tube into a 1.5 mL tube. Combine up to 400 uL, in each tube. Note: phase-lock tubes can also be used here to improve recovery.
    - Carefully pipet and discard bottom oil phase after droplets float to the top. Press a P1000 down to its first stop, push through the droplets to the bottom of the tube, press down to the second stop to expel any droplets, then wait several seconds for the droplets to float back up to the droplet layer, and finally aspirate out the oil. You do not need to remove every last bit of oil - just remove most of it.
    - Add 50 uL of TE buffer for each 100 uL of PCR reaction combined in the 1.5mL tube.
    - In a fume hood, add 175 uL of chloroform for each PCR reaction in the tube. (If there are 4 PCR reactions in a tube than contents will be: <400uL PCR reactions, 200uL TE, 700 uL chloroform).
    - Vortex at maximum speed for 1 min.
    - In a centrifuge, spin down at 15,500 x g for 10 min.
    - Remove upper aqueous phase by pipetting, avoiding the chloroform phase.
    - Transfer this to a clean 1.5mL tube (this is the DNA).

- Proceed to column or SPRI bead cleanup (Beckman) for the recovered DNA.

9. Size selection.
    - The amplicons will often be mixed with undesired lower-molecular weight assemblies. Removing these using size selection will increase final yield. Choose of of the following three approaches, ordered from highest yield to lowest yield:
        - Pippin Prep (Sage Science).
            1. Follow manufacturer's protocol (calibration, checking currents, loading, etc…)
            2. Make sure to allow for a range broad enough to include every member of the library, yet narrow enough to exclude some of the shorter non-specific products (+/- 100 bp is usually fine).
            3. Collect the eluted product and column cleanup using a Zymo Clean & Concentrator -5 (Zymo Research).
        - or 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.
        - or No size selection.
            1. Make a dilution of ePCA and use this as template for the re-amplification.
10. Re-amplification.
    - Amplify ePCA products using Kapa HiFi HotStart ReadyMix (2X) (KAPA Biosystems).
        - 0.2 - 2 uL template
        - 1 uL 10 uM FWD assembly primer
        - 1 uL 10 uM REV assembly primer
        - 25 uL Kapa HiFi HotStart ReadyMix (2X) (KAPA Biosystems)
        - UltraPure Distilled Water (Invitrogen) to 50 uL
        - TOTAL: 50 uL
        - PCR protocol:
            1. 3 min 95ºC initial denaturation
            2. 15 sec 98ºC denaturation
            3. 20 sec 58ºC annealing
            4. 45 sec 72ºC extension
            5. Go to step 2, determine cycles using qPCR.
            6. 3 min 72ºC final extension
    - Column purify re-amplified products using a Zymo Clean & Concentrator -5 (Zymo Research).
    - Check size distribution on gel or tapestation.
    - Quantify DNA and proceed to downstream applications.