Purpose: Drive library fragments to ISPs (beads) and populate the beads with emPCR. Quantify the yield using the Qubit..

All of the libraries gave varying degrees of fragments in a usable size range. Groups 1 and 2 will work together to process Library 1 on the OneTouch-ES robot and Groups 3-5 will use the IKA Turrax on the Group 5 library. That is, we will process one library from the 250bp mean (Libraries 1-3) and one from the 300bp mean (Libraries 4-6, remember that 6 was from DNA that Deepthi Chaturvedi prepared). The advantage of the OneTouch is that no pipetting has to be done once the mixture is made. The advantage of the Turrax is that the PCR conditions can be modified to compensate for unusual templates.

We first have to determine how much of the first-stage library to use, as discussed in class. Then we have to set up the reaction conditions for the PCR (with the A' and P1' primers) and form an oil emulsion.

Protocols

Library Dilutions

1. For each library calculate the required dilution to obtain an optimal ISP loading.

Library	Stock Conc (in Molecules per ul)	ul stock for $7.78*10^9$	Volume buffer	
1	4.21E+09	1.8484	498.1516	
2	2.17E+08	35.8261	464.1739	
3	5.13E+07	151.6540	348.3460	
4	2.96E+06	2630.1555	-2130.1555	Note: don't use
5	2.63E+08	29.6212	470.3788	
6	4.18E+07	186.0354	313.9646	

a. Once the correct dilution is made, label the tube and store it on ice.

Turrax version of emPCR protocol

2. Add 9ml of the emulsion oil to a DT-20 tube (it is sticky so pipette slowly to make sure fill delivery occurs). Place on ice until ready to use.
    a. The ISP particle tube contains 100ul of particles in a glycerol buffer. Vortex at the highest speed for 1 minute.
    b. Prepare the PCR-fragment mixture (the aqueous part). The final volume will be 1ml.
        i. To a 1.5ml LoBind tube, add 582 ul of NF-H2O (nuclease-free water)
        ii. Add 200ul of the 5X PCR reagent mix (violet cap)
            1. Vortex for 5 second, quick-spin to collect
        iii. Add 100ul of 10X PCR Buffer (brown cap)
            1. Vortex for 5 second, quick-spin to collect
        iv. Add the 100ul of vortexed ISPs.
            1. Vortex for 5 second, quick-spin to collect
        v. Add 18ul of the FINAL DILUTION of the library
            1. Vortex for 5 second, quick-spin to collect
3. Place the DT-20 tube that contains the oil on the drive, twist it to lock it in place.
    a. Press START to get the Turrax drive going (give it 2-3 seconds)
    b. Take the label off the top of the tube (keep it) and use a P1000 to dispense the PCR solution into the oil (take your time) – then re-cover the hold with the label.
    c. *Continue the mixing process* for 5 minutes, then stop the drive and move the tube to ice.
    d. Let the emulsion sit on ice for 5 minutes
    e. Transfer the emulsion to a reservoir that is on ice (pipette slowly)
    f. Using an 8multi-channel pipette with tips that have been cut off with a razor blade about 1/5th of the way up (so the thick emulsion can be delivered), transfer 100ul of the emulsion to each of the wells <u>of a 96-well plate that is sitting on ice</u> (pipette slowly, you will probably not fill all of the plate). Scavenge the last of the emulsion from the reservoir with a single channel pipette (tip cut off) and dispense into unfilled wells.
        i. Try to deliver the same volume to all tubes, which means slow rates of input and output of the emulsion.
        ii. Don't rest the tip against the side of the plate (it will contaminate the PCR machine).
    g. Carefully cap the wells (make SURE the caps are firmly seated on the tubes).
4. Start the thermocycling using the following parameters (protocol 'emPCR')

a. Ramp rate is on Maximum, cover is at 100 (time is ~3 h)

Stage	Step	Temperature	Time
Hold	Denature	94°C	6 min
Cycle (40 cycles)	Denature	94°C	30 sec
	Anneal	58°C	30 sec
	Extend	72°C	90 sec
Cycle (5 cycles)	Denature	94°C	30 sec
	Extend	68°C	6 min
Hold[5]	—	10°C	∞

b. Post-PCR is a stopping point - this can be stored for up to a week at 4C.

5. The ISPs have to be recovered from the emulsion (very messy). First check for wells with failed emulsions – they will have a clear top phase, while we only want the cloudy ones - make sure to avoid these wells)
   a. Collect the emulsion <u>from the good wells</u> in the plate into a reservoir – then transfer into up to six 1.5ml LoBind tubes.
      i. Use the 8-channel pipetter with tips cut off. Pipette slowly.
      ii. Keep the plate and reservoir, you will rinse them with BREAKING solution.
   b. Centrifuge balanced tubes at 15,500 x g for 2 minutes.
      i. Remove the top, clear layer (the oil) from each tube – put this in the ORGANIC WASTE bottle. Keep the lower white part!
   c. **Breaking solution**: mix 2ml of Recovery solution with 6 ml of butanol in a 15 ml tubes.
      i. Vortex on high for 1 minutes (or until a complete emulsion is formed).
   d. Add 1ml of the Breaking solution to each 1.5 ml tube
      i. Add 50ul of Breaking solution to the first column of the PCR plate, pipette up and down and transfer to the next column and so on until all of the PCR tubes are rinsed, transfer to the reservoir to rinse it, then dispense to the 1.5 ml tubes evenly
      ii. Vortex tubes for 30 seconds – invert and make sure the whole solution is mixed, if not vortex some more.
   e. Centrifuge balanced tubes at 15,500 x g for 2 minutes. Make sure the tubes look like this:  with 2 phases and a white interphase layer.
   f. Remove the TOP layer (the butanol) from each tube – pipette as close to the interphase as you can. Discard the butanol in ORGANIC WASTE bottle.

g. Wash each sample with 1 ml of **Recovery solution** (NOT Breaking Solution!!)
    i. Add 1 ml of Recovery solution to each tube
    ii. Vortex on high for 30 seconds
    iii. Centrifuge at 15,5000 for 3 minutes
    iv. Remove the top layer to ORGANIC WASTE
    v. Remove as much of the interphase as you can without losing much of the layer beneath.
    vi. You should have ~100ul left in each tube.

h. Pool the material from these tubes into a new single tube using the same tip for all of them, pipetting slowly and making sure no pellet is left behind, in the bottom of any of them (this is the spheres, which are clear and which you DO NOT WANT TO LOSE).
    i. Add 200ul of Recovery Solution to one of the old tubes and rinse the sides carefully, transfer this to the next tube and so on until all have been rinsed – add the final rinse to the tube containing the bulk of the reactions.
    ii. Repeat the step above. The volume in the tube should be ~800ul.

i. Add Recovery Solution (NOT Breaking solution) so the final volume is 1.5 ml - this should be ~700 ul, but make sure you do not over-flow the tube.
    i. Vortex the tube on high for 30 seconds.
    ii. Centrifuge at 15,500 x g for 3 minutes.
    iii. Remove the top layer to the ORGANIC WASTE.
    iv. Remove as much of the interphase as you can without losing the material in the bottom layer – you want ~100 ul remaining when you are done.

j. **Wash Solution step**: Gently pipette the 100 ul up and down and remove it to a fresh 1.5ml LoBind tube. Keep the first tube and keep the transfer tip on its pipette (the spheres are sticky and some are on still in the tube and on this tip)
    i. Using a different pipette and tip, add 100ul Wash Solution to the original sample tube and then using the original tip/pipette, rinse the tube carefully – transfer this to the new sample tube. You should have ~200ul of solution.
    ii. Add 1000ul of Wash Solution to the sample tube and vortex on HIGH for 30 seconds
    iii. Centrifuge at 15, 500 x g for 3 minutes
    iv. Remove the supernatant and as much of the interphase as you can without losing much of the lower layer to the ORGANIC WASTE. You should have ~100ul left in the tube.
    v. Repeat the 1000-ul wash, vortex, spin and cleanup steps above.

Note: the ISPs can now be stored at 4C for up to one week.

Perform the Qubit Test on the ISPs (this gives the number of spheres with targets which should be from 10-30%).

6. The Qubit is a 2-wavelength fluorometer that allows you to compare the amount of one adaptor to the other.
    a. Power on the Qubit 2.0 Fluorometer by plugging it in (make sure the USB drive is inserted first)– we have version 3.0 so you don't need to worry about upgrades. You these screens (if you touch ION in the first case)

    b. There is a calculator for the factors in the Protocol section for class use (Qubit 2.0 Easy Calculator Microsoft Excel Spreadsheet).
    c. You will find one tube containing 200ul of the Control FAM CS (calibration standard) and one tube containing 200ul of the Control Cy5 CS sitting on ice.
        i. Pulse spin to collect all reagent in the bottom of the tube.
        ii. Press the IonFAM button, insert the tube, close the lid, press READ
        iii. When the light turns red record the value (~5 seconds), remove the tube
        iv. Press Home, then press the IonCy5 button, insert the Ion Cy5 CS tube, close the lid, press READ
        v. When the light turns red, record the value, then remove the tube.

Qubit Calibration Factor Calculation

Calibration Standard	RFU	Calibration Factor
FAM Calibration Standard		#DIV/0!
Cy5 Calibration Standard		

Templated Signal Ratio

	Raw RFU Value		Background RFU (Negative Control Tube)			
Sample ID	IonFAM	IonCy5	IonFAM	IonCy5	Conversion Factor*	Percent Templated ISPs
						#DIV/0!
						#DIV/0!
						#DIV/0!
						#DIV/0!

* Conversion factor can be found on Ion Community website (www.iontorrent.com/qubit_files/) and is Template Kit lot specific.

Green Cells = Raw RFU values of FAM and Cy5 Calibration Standards supplied in the Ion Sphere Quality Control Kit
Red Cells = Raw RFU values measured in Section 3: Ion Sphere Particles Quality Control Sample Measurement
Purple Cells = Raw RFU values measured for negative control in Section 3: Ion Sphere Particles Quality Control Sample Measurement
Blue Cells = Template kit lot specific conversion factor

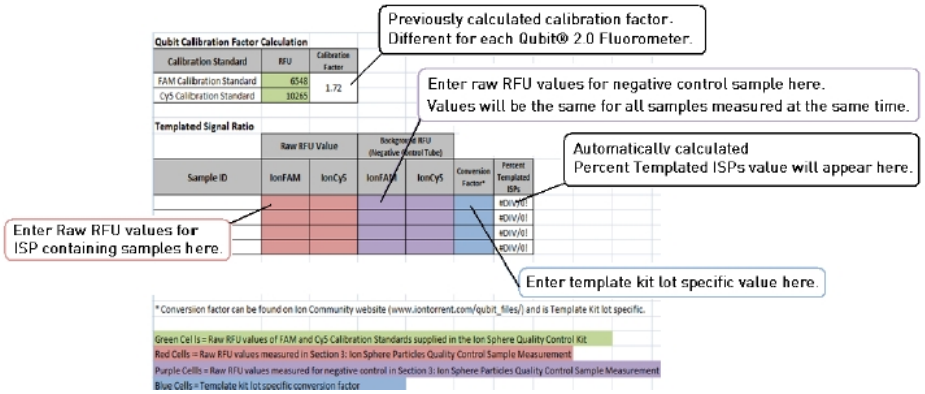
        vi. Check the volume of your ISPs, and adjust to 100ul using Wash Solution, mix well (vortex and quick-spin).

vii. Transfer 2ul to a 200ul PCR tube.
viii. Add 19ul of Annealing Buffer to the PCR tube, mix by pipetting
ix. Add 1ul of Ion Probes to the PCR tube, mix by pipetting

x. Put tube into thermocycler with these conditions

Temperature	Time
95°C	2 min
37°C	2 min

xi.
xii. Add 200 ul QCWB (quality control wash buffer – NOT THE SAME AS TEMPLATE WASH SOLUTION!!!).
xiii. Vortex on high, centrifuge at 15,500 x g for 90 seconds; remove all but 10ul of material - retain the material in the sample PCR tube.
    1. Put 10ul of solution into another PCR tube and use this as a visual aid when removing the QCWB so you know how much to leave in the sample tube.
xiv. Repeat wash twice more
xv. Add 190ul of QCWB. Pipette up and down 4-5 times, verify that the volume is 200ul (adjust upwards if it is low).
    1. Transfer to a Qubit assay tube (SAMPLE tube)
    2. Place 200ul of QCWB in a separate Qubit assay tube (NC tube – negative control).
xvi. Turn on the Qubit, USG drive inserted, touch ION, then IonFAM
    1. Insert the SAMPLE tube, close the lid, press READ, when the light turns red record the value (~5 sec); press HOME
    2. Press HOME, press Ion CY5, press READ, when the light turns red record the value. Remove the tube, press HOME.
    3. Repeat for the NC tube.
xvii. Evaluate the data by putting the recorded values into the appropriate fields in the calculator.



xviii. The acceptance criterion for un-enriched spheres is 10-30%. Record the value for this set of spheres. If within these limits we will enrich next time, and continue to sequencing.

The OneTouch emPCR method

In this case the robot takes care of creating the emulsion, dispensing it to the plate, performing the PCR, recovering the IPS post-PCR, and washing them. They end up in two tubes so the mini-microfuge will be balanced.

7. Make sure the instrument is set up, the tube on the left front should be half-full of oil – if not complete the following steps:
    a. Invert the OneTouch Oil stock bottle 10 times, refill the tube, cap the tube and invert it 10 times , then remove the cap and seat firmly on the instrument
        i. If the sipper tube touches anything during the process, replace it.
    b. The Recovery solution may need to be warmed slightly before it clears (hold it under warm water and swirl). Invert the bottle 10 times, fill the tube on the right half-full. If combining solutions, cap the tube, invert 10 times to mix, remove the cap and seat the tube firmly.
        i. If the sipper tube touches anything during the process, replace it.
    c. Install 2 OneTouch collection tubes in the mini-centrifuge, connected with the router, then close the lid
    d. Insert a PCR plate and close the latch.
    e. Check that the Waste container is not full.
8. Collect a 1.5 ml LoBind SAMPLE tube. Add reagents in the following order
    a. 595ul of NF-H2O
    b. 200ul of OT-Reagent Mix (violet cap)
        i. Vortex for 5 sec, pulse-spin
    c. 100ul of OT-Enzyme mix (brown cap)
        i. Vortex for 5 sec, pulse-spin
    d. 5ul of your DILUTED library stock.
        i. Vortex for 5 sec, pulse-spin

ii. You should now have 900ul of solution

e. Take the OT-ISP tube (with 100ul) and Vortex on HIGH for 60 seconds, pulse spin
   i. Add ISPs to the SAMPLE tube, pipette up and down 5-6 times to mix
f. This solution should sit at room temperature for 10 min before proceeding to the next step.

9. Collect a OneTouch Reaction tube and filter, and the emulsion oil. Set up the Reaction tube so that it cannot tip while you add solutions to it (a 15-ml Falcon tube in a rack works well).

   a. Add the 1000ul of SAMPLE solution to the bottom of the Reaction tube.
   b. Carefully overlay the SAMPLE solution with emulsion oil - start with 100ul and let it slide slowly down the side from near the top of the aqueous layer. When you get more oil use a new tip! Once you have added several hundred ul you can start adding more at a time.
   c. Minimize bubbles, fill to the top.

   d. Attach the filter firmly – make sure it is evenly seated.
   e. SLOWLY invert the tube (try not to cause any mixing of the layers)
   f. Insert the 3 prongs of the filter into the 3 ports of the OneTouch instrument, PUSH DOWN to seat the filter into the ports.

10. Running the OneTouch – from the drop-down menu selected the ASSISTED RUN option, follow the directions, hitting NEXT as they tasks are completed.
    a. Run time is 4 hours
    b. Once the run is completed you do have to give a command – there is a command for the centrifugation step - you <u>touch</u> the Centrifuge Sample command (it is not in a step-box but looks like it is between two such steps, it will have a small red light that will turn green when you touch it).
       i. If you hit NEXT <u>without</u> centrifuging you will have to do it by hand, just like the other group (groan). Here is what you do:
          a. Remove the 2 Recovery Tubes.
          b. Pipet the solution up and down in one Recovery Tube to resuspend the solution.
          c. Transfer the solution from *one* Recovery Tube to a new 1.5-mL LoBind Tube.
          d. Spin the LoBind Tube at 15,500 × g for 3 minutes.
          e. Remove all but 50 µL of solution from the LoBind Tube.
          f. Pipet the solution up and down in the other Recovery Tube to resuspend the solution.
          g. Transfer the solution from the other Recovery Tube to the 1.5-mL LoBind Tube.
          h. Spin the LoBind Tube at 15,500 × g for 3 minutes.
          i. Remove all but 100 µL of solution from the LoBind Tube.
       ii.
    c. If you DID centrifuge properly, carefully retrieve the 2 Sample Recovery tubes from the mini-centrifuge.

i. Remove all but 50 ul from each tube (use a blank loaded with 50 ul of solutions to have a visual aid to the level needed). Use the same pipette tip for both.
ii. Do remove any white fluffy material that you see in the tubes.
iii. With a clean tip, pipette each solution up and down repeatedly to disperse the pellets (use the same tip for both tubes).
iv. Combine the two samples in a single 1.5ml LoBind tube. Add 1ml of OT-Wash Solution , vortex on high for 60 sec, then centrifuge at 15,500 x g for 3 minutes. Remove all but 100ul from the tube.
v. Disperse the ISPs through the 100 ul by pipetting up and down 5-6 times. Remove 2ul to a 200ul PCR tube and proceed to the QUBIT TEST (see step 6 above) of pre-enriched ISPs.
vi. Store the remainder of the ISPs at 4C until next week when we will enrich and sequence.

d. Clean the instrument
i. Wipe the lid, remove and discard the router and clean the injector tubing.
ii. Remove and dispose of the PCR plate
iii. Remove and dispose of the reaction tube/filter setup