Purpose: Enrich ISPs, perform second Qubit assessment, Load Ion Torrent chip.

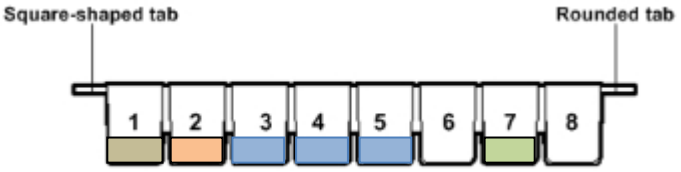
We have 2-fold goals with enrichment: to pull the ISPs that do not have any target attached away from those that do, and then to melt off the complementary strand of the target attached to the ISP, so that we can use the attached strand as a sequencing template. The first part is done using the biotin that is attached to the P1 primer (the complementary strand) – a streptavidin molecule attached to a magnetic bead will hold the biotin, the nucleotide to which it is attached, the DNA molecule in which that is incorporated, the duplex in which that strand is H-bonded AND the ISP to which that duplex is attached. Everything else will be washed away. Then the duplex will be melted (using NaOH, not heat) and the biotin-containing DNA strand will be retained on the bead while the ISP with its template will be washed away (we will keep the ISP, of course). Once the ISPs have been enriched we repeat the Qubit test for ISP-template occupancy. If the ISPs pass then they are loaded on the 314 chip with primer and polymerase and placed on the sequencer.

Very Important!!! The ION Torrent sequencer works by detecting changes in the pH, so control of the pH of the reference solutions is essential for success. Even the amount of CO_2 that water absorbs passing through the air changes the pH enough to ruin the chemistry. Because of this, we collect 18-MΩ water immediately before using it, rinse all solution bottles with that water immediately before filling them, make fresh 0.1M NaOH immediately before adding it to the Wash solution, and maintain the solutions under Argon gas once they are made.

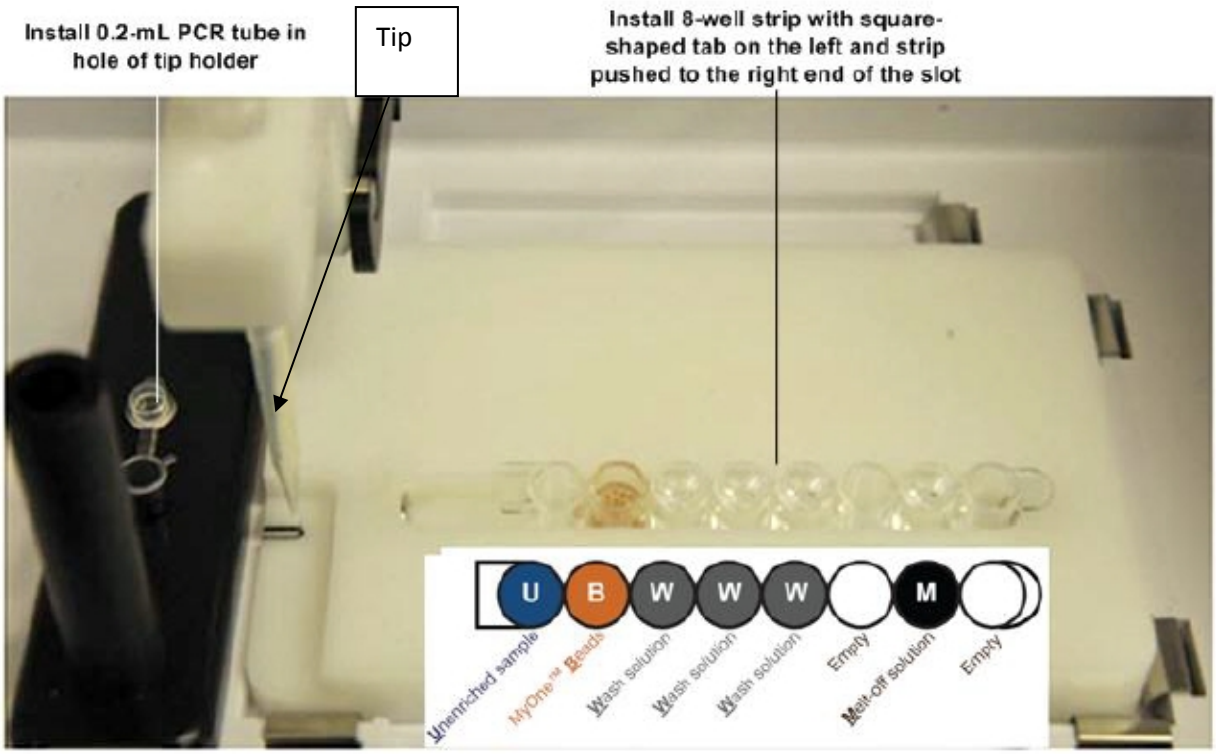
Protocols

Enrichment with the ES unit

1. Prepare Melt-off solution (fresh each day)
    a. Nuclease-free H_2O (NF-H_2O) 865 ul
    b. 1M NaOH 125 ul
    c. 10% Tween 20 in NF-H_2O 10 ul
    d. Mix. Check final volume is 1000 ul
2. Collect a tube of MO beads from the ice bucket (this 1.5 ml LoBind tube contains 13ul of Dynal MyOne Streptavidin beads in 130ul of Wash Solution).
    a. Resuspend the beads by pipetting up and down 10 times to mix. Try not to leave residual solution on the sides of the pipette tip.
    b. Place the tube in a magnetic holder for 2 minutes
    c. While in the holder, open the tube, remove the liquid (discard)
    d. Remove the tube from the holder, add 130 ul of MOW (MyOne Wash buffer) to the tube, pipette up and down 10 times.
    e. Place this solution in Well 2 of the ES strip shown below.



3. Since we stored the template ISPs we perform the following
    a. Centrifuge the tube at 15,500 x g for 3 minutes
    b. Carefully remove all but the bottom 10ul (use a comparison tube with 10ul to estimate the volume)
    c. Add 90 ul of OneTouch Wash Solution. Pipette up and down 10 times to mix.
        i. Verify that final volume is 100ul.
4. Add the 100 ul of ISPs to Well 1 of the ES strip.
    a. Verify that Well 2 contains the MyOne beads
    b. In Wells 2-5 add 300ul of OneTouch Wash Solution.
    c. Well 6 is empty
    d. Well 7 contains 300ul of your freshly made MeltOff solution
    e. Well 8 is empty
5. The ES unit is shown below – note the position of the strip, the position of the PCR tube, and the tip on the Loader Arm.
    a. Place the strip all the way to right in it's slot.
    b. Place a new tip on the end of the Tip Loader arm.
        i. Remove the arm from its cradle, align the metal fitting with the new tip and press down on the tip arm firmly for ~1 second to install the tip, then lift the tip arm straight up.
        ii. Return the tip arm to the cradle by tilting it back and aligning the pins with the notches, then lowering the arm into position.
        iii. Rock the arm forward into its working position.
    c. Insert at 0.2 ml PCR tube (lid up) into its holder.



6. Perform the enrichment in the following steps (this will take ~35 minutes).
    a. Initialize the ES by turning it on – it will show 'rdy' when it is ready.
    b. Gently pipette the contents of Well 2 up and down 10 times (no bubbles!)
    c. Press START/STOP once (if you press it again at any time it will end the run and you cannot return to an intermediate step).
    d. The instrument will beep every 60 seconds when processing is complete and display 'End'. You can press Start/Stop at this point to stop the beeping.
        i. Immediately cap the PCR tube – this is your sample!! Verify that it has >200ul of solution in it (it should be close to full). Remove to an ice bucket. You must wash to neutralize the pH at once – step 7 below.
            1. If the PCR tube is not close to full check Well 8 – if it contains solution collect it to the same PCR tube and neutralize as described below, recalibrate the OneTouch ES and repeat the purification.
        ii. Check the wells in the strip: Well 1 should have <2ul of bead-containing solution, Well 2 will also have a trace amount of residual beads and Wells 2-5, 7 have residual reagents (less than starting).
    e. Instrument clean-up: remove the used tip by twisting the tip counterclockwise and pulling it down. Remove and discard the 8-well strip.
7. Wash the enriched ISPs
    a. Spin the PCR tube with the enriched ISPs at 15,500 x g for 90 seconds
        i. Be sure to use the correct tube adaptors or your tube will be crushed along with your hopes.

b. If there is a brownish color in your pellet, resuspend the pellet by pipetting up and down gently 10 times.
    i. Place the tube in a magnetic rack, let sit for 4 minutes
    ii. Transfer the solution to a fresh 0.2 ml PCR tube (discard the old tube)
    iii. Repeat step 7a using the transferred solution – this is your sample!
c. Remove all but 10ul of the solution from the tube (put 10ul in another tube and use it for comparison). Pipette from the top, do not disturb the pellet!
d. Add 200ul of OneTouch Wash Solution and pipette up and down 10 times to resuspend.
e. Centrifuge at 15,500 x g for 90 seconds.
f. Remove all but 10 ul of the solution from the tube, be careful not to disturb the (invisible) pellet.
g. Add 90ul of OneTouch Wash Solution. Verify that the final volume is 100ul (adjust if low).
    i. Pipette up and down 10 times to resuspend the ISPs.
    ii. Remove 2ul to quantify on the Qubit.
    iii. Store for up to 1 week at 2-8C.

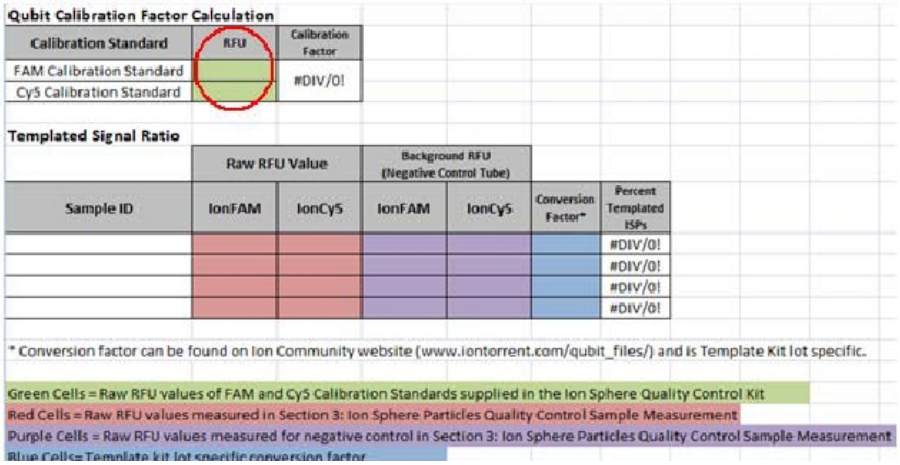
Perform the Qubit Test on the ISPs

1. 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

2. Check the volume of your ISPs, and adjust to 100ul using Wash Solution, mix well (vortex and quick-spin).
    a. Transfer 2ul to a 200ul PCR tube.
    b. Add 19ul of Annealing Buffer to the PCR tube, mix by pipetting
    c. Add 1ul of Ion Probes to the PCR tube, mix by pipetting up and down
    d. Put tube into thermocycler with these conditions
    e.

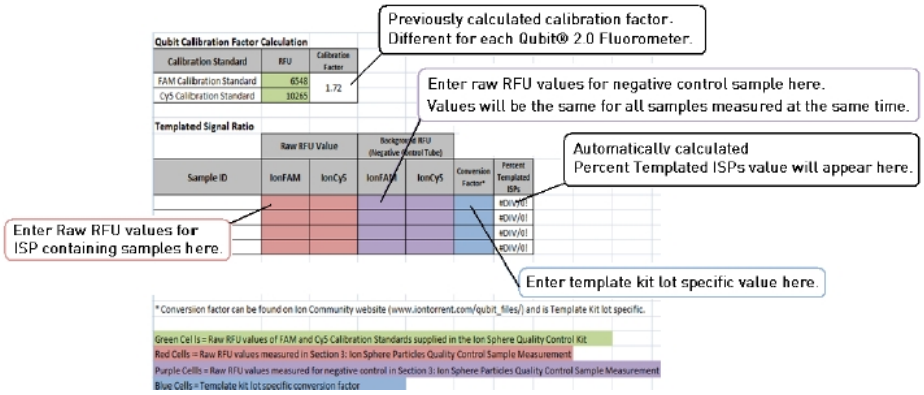
Temperature	Time
95°C	2 min
37°C	2 min

    f. Add 200 ul QCWB (quality control wash buffer – NOT THE SAME AS TEMPLATE WASH SOLUTION!!!).
    g. 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.
        i. 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.
    h. Repeat wash twice more
    i. Add 190ul of QCWB. Pipette up and down 4-5 times, verify that the volume is 200ul (adjust upwards if it is low).
        i. Transfer to a Qubit assay tube (SAMPLE tube)
        ii. Place 200ul of QCWB in a separate Qubit assay tube (NC tube – negative control).
3. Turn on the Qubit, USB
4. drive inserted, touch ION, then IonFAM
    a. Insert the SAMPLE tube, close the lid, press READ, when the light turns red record the value (~5 sec); press HOME

i. Press HOME, press Ion CY5, press READ, when the light turns red record the value. Remove the tube, press HOME.

b. Repeat for the NC tube.

5. Evaluate the data by putting the recorded values into the appropriate fields in the calculator.



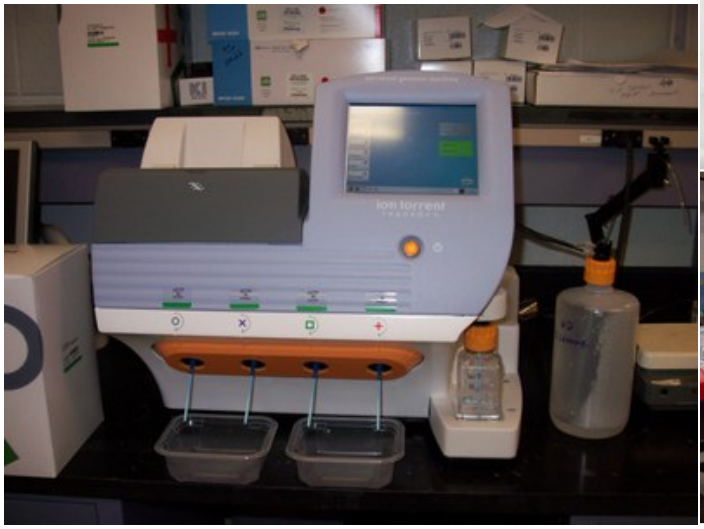
6. Record the values for your spheres. The acceptance criterion are as follows

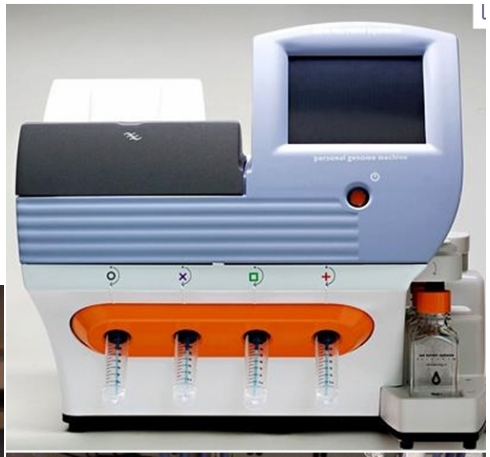
a. Un-enriched spheres: 10-30%.

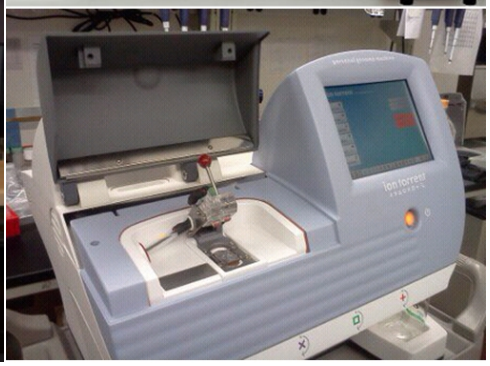
b. Enriched spheres: > 50%

Sequencing

Sequencing your library involves instrument/reagent preparation and loading your ISPs on the prepared chip.







Instrument Preparation

You will rinse bottles and make fresh Wash solutions, make fresh dNTPs and make sure the system is under Argon and without leaks.

1. Preparation:

    A. From the 10M NaOH stock prepare 10ml of 1M NaOH using 18MΩ H_2O. From that 1M NaOH stock prepare 10ml of 0.1M NaOH stock using 18MΩ H_2O.

    B. Locate bottles W1 (250ml), W2 (2-L) and W3 (250ml) and Cleaning (250 ml).

    C. An old chip should be taken out and clamped in (no gloves, be sure to ground yourself!).

    D. Check that the level of Argon in the tank is at least 500psi.

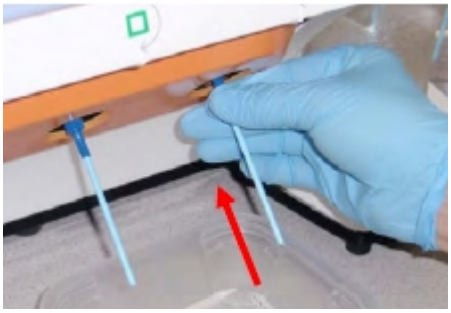
2. Cleaning Weekly - Note: we will do this the morning of the lab – but FYI, once per week the instrument is cycled through a chlorite regimen. In between simple water rinses are sufficient, see the manual for full details. Remove any bottles that are attached to the PGM, but NOT the sippers. Make sure an old chip is clamped in.
    a. To 1L of 18MΩ H_2O add 1 cleaning tablet (water purification tablets such as REI provides). Let sit for 10 minutes, no more than 3 hours.
    b. Add 1 ml of 1M NaOH and mix
    c. Filter in a 0.22 or 0.45 vacuum filter device
    d. Transfer 250ml of filtrate to the Cleaning bottle and place at the W1 position on the PGM.
    e. Select the Cleaning procedure from the Menu and follow the instructions
    f. When complete, remove the Cleaning bottle, rinse the sipper with 18MΩ H_2O from a squirt bottle and install a fresh Cleaning bottle containing 250 ml of 18MΩ H_2O in its position.
3. Cleaning Daily. Remove any bottles that are attached to the PGM, but NOT the sippers. Make sure an old chip is clamped in.
    a. Empty each bottle and rinse 2X with 100 ml of fresh 18MΩ H_2O.
    b. Select Clean PGM protocol
    c. Add 250 ml of fresh 18MΩ H_2O to a 250ml Cleaning bottle, attach to the W1 position
    d. Follow the instructions on the screen – when prompted to remove the W2 bottle rnse the sipper from a squirt bottle, rinse the Clean bottle with more 18MΩ H_2O, fill with fresh 18MΩ H_2O and reattach it to the instrument.
    e. Press NEXT to return to the Main Menu – initialization can proceed.
4. Wash Solutions:
    a. Rinse W2 bottle 3X with 200 ml of fresh 18MΩ H_2O.

i. Insert a magnetic stir bar, place on a stir plate near the PGM and insert the Argon tubing in the bottle, adjusting the flow to 0.5L/min.
    1. Let sit for 5 minutes
    2. Shut off the Argon flow and remove the tubing WHILE capping the bottle.
    3. Take the bottle to the 18MΩ H_2O water dispenser and fill to the line (1978 ml). Hold the top as close to the dispenser as possible. Recap as soon as filled.

ii. Replace the bottle on the stir plate, replace the argon tubing and set the flow for 0.5L/min.
    1. Turn on the plate sufficiently to have a small whirlpool form.
    2. Using a 25 ml serological pipette, add 22 ml of W2 solution to the bottle. Let stir 1 minute.

b. Rinse W1 and W3 bottles each with 3X 50 ml of fresh 18MΩ H_2O.
    i. To W1 add 350ul of 0.1M NaOH and cap the bottle.
    ii. To W3 bottle
        1. Add 5 ml of 10X W3 solution
        2. Add 45 ml of 18MΩ H_2O.
        3. Cap the W3 bottle
        4. to the fill line (at 1978 ml, marked in Sharpie)

5. Initialize the instrument (30 minutes)
    a. One the PGM press Init PGM – it will check the Argon pressure, if it passes press NEXT.
    b. Wearing gloves, install a new sipper (long gray tube with a Luer lock on one end) in the cap on the W2 position of the PGM (it must not touch any surfaces and be careful not to cross-thread).
        i. Install the Wash 2 bottle.
        ii. Press NEXT
    c. Install new sippers (short gray tubes) in the caps for W1 and W3, and immediately attach the bottles. Press NEXT.
    d. The instrument checks for leaks, fills bottles and performs pH calibration internally. You can prepare the dNTPS while this is going on.
6. Make dNTP solutions wearing gloves
    a. Vortex the thawed dNTP stock tubes, quick-spin
    b. Label 4 new conical tubes (one per nt)
    c. Transfer 20ul of each dNTP to the bottom of the correct conical tube.
7. Once Wash solutions are initialized follow on-screen prompts to install dNTP tubes

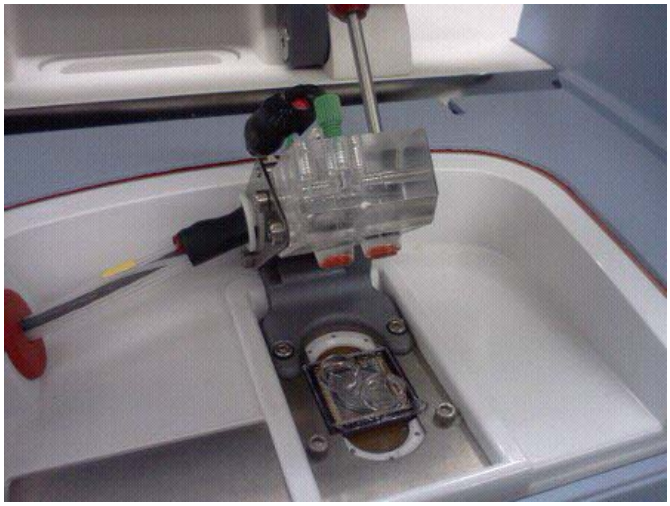


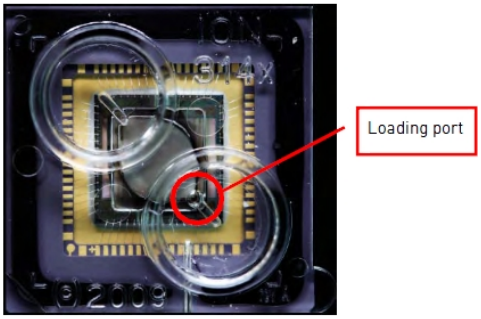
a. Remove the old sippers and discard
b. Change gloves
c. Install the new sippers with a push rather than a twist (they must not touch anything)
d. Attach each conical tube to the correct location and tighten until very snug
    i. The instrument will fill each tube to ~40 ml, enough for 2 runs.

8. Follow on-screen prompts to complete initialization, and once complete hit NEXT to return to the main menu
9. The ISP-Chip preparation can be performed while the instrument is being set up.

314 Chip Preparation –

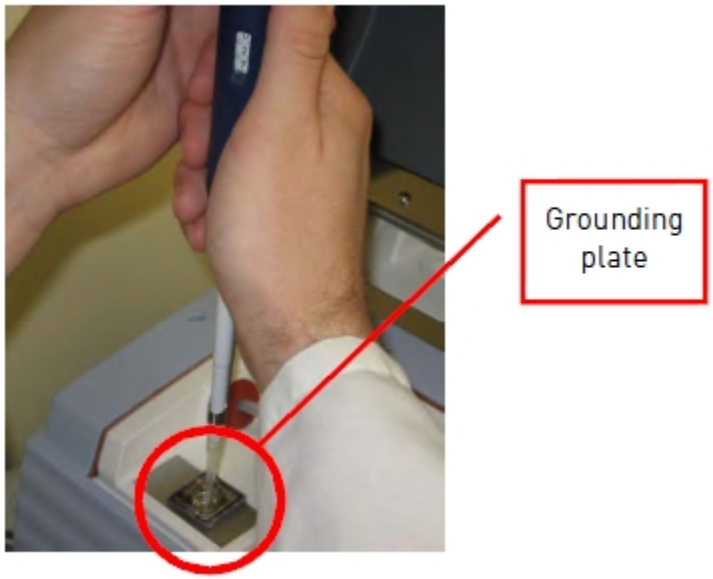
You will add a small fraction of Control ISPs (this calibrates the analysis software, as these spheres contain known templates) to your own spheres. Then you will anneal the sequencing primer to off of the templates, add the sequencing reagents, then seat the beads in the 314 chip wells.





1. Verify that you have 100ul of template IPS. Vortex the tube for 10 seconds.
    a. Quick-spin to collect the solution
2. Transfer 50ul of the template ISPs to a new 0.2 ml PCR tube.
3. Vortex the Control Ion Sphere tube for 10 seconds, quick- spin to collect the solution
    a. Transfer 5ul of the Control ISPs to your tube of template ISPs.
4. Add 150 ul of AB solution (annealing buffer)
    a. Mix by pipetting up and down 10 times (try not have residual volume in your tip when you are done).

5. Centrifuge for 2 minutes at 15,500 x g (balance, use the correct adaptors to avoid crushed-tube syndrome).
    a. Remove the supernatant from the top – leave just under 9ul of solution in the bottom of the tube (use a parallel tube with 9ul in it for visual comparison of levels).
    b. Measure the remaining volume - if it is LOW, add Annealing Buffer to adjust.
6. Add ul of SP (sequencing primer) to the ISP sample, mix by pipetting up and down ten times, try not to have residual volume in the tip when you are done.
7. Call up the PGMseq protocol on the thermocycler - here is the program if you can't find it
    a. Heated Lid ON
    b. 95C 2 min
    c. 37C 2 min
    d. Note: the tube can remain at RT (room temperature) while you perform the Chip Check step.
8. Chip Check - remember that the chips are sensitive to any type of electrostatic discharge, which builds up easily on surfaces and people. Therefore you should never place a chip directly on a bench surface – use either the chip rotor adaptor or the PGM grounding plate for setting it down. Do NOT WEAR GLOVES when handling the chip!!!
    a. Press Experiment on the main menu and follow the prompts until it asks for a chip to test.
    b. Remove a chip from the envelope (keep the envelope so you can scan the bar code on it. Also this is a good way to save the chip when you are done).
    c. Place the chip on the grounding plate on the PGM
    d. Using a Rainin SR-L200F (low retention) tip, carefully pipette 50ul of 100% isopropanol into the large port on the chip.



        i. If some liquid flows through the other port, pipette it off.
    e. Using a Rainin SR-L200F tip, carefully pipette 50ul of Annealing Buffer into the large port
        i. Aspirate off the displaced isopropanol.

f. Using a Rainin SR-L200F tip, carefully pipette 50ul of Annealing Buffer into the large port
    i. Aspirate off the displaced isopropanol.
g. Ground yourself by touching the grounding pad (no gloves!)
    i. Place the chip in the chip holder and clamp it in place.
h. Scan the bar code on the envelope.
i. Press Chip Check.
    i. If this fails, open the clamp, research the chip, close the clamp and try again. If it still fails repeat the washing step (isopropanol followed by Annealing Buffer). If it still fails, try with a second chip.
    ii. If the chip passes, press NEXT (the system washes it with Wash 2 and calibrates the sensor response)
        1. If there is a leak, press ABORT
    iii. Remove the chip a prompted, pipette 50ul of Annealing Buffer into the large port, aspirating off any extra than emerges on the other side.

9. Prepare and Load the templated ISPs
    a. Remove the PCR tube from the thermocycler
    b. Add 1ul of Sequencing polymerase, pipetting up and down to mix (try to have no residual volume in the tip when you are done).
        i. Incubate at RT for 5 minutes
        ii. Put the tube in the small 'floater' and place in the 40MHx sonicator. Turn on for 10 seconds.
        iii. Remove the tube to the bench.
    c. Place the 314 chip in the minifuge adaptor (no gloves). Mark your chip with a Sharpie so you don't confuse it with the balance tube.

    d. With a Rainin SR-L20F tip carefully (very slowly and with the pipette vertical) deposit 7ul of the sample into the large port of the chip.
        i. DO NOT push all of the sample out the bottom of the tip! This will introduce bubbles. In this case a small residual volume in the tip is desirable.
        ii. Remove any solution that comes out the other port (same pipette tip, reserve solution)
    e. Seat the adaptor in the minifuge, and balance with a used chip on the other side.

i. Centrifuge for 4 minutes

f. Remove the adaptor holding the chip and place on the bench.

i. Using the reserved tip, collect fresh 7ul of library sample and deposit on the chip via the loading port (very slowly, with the pipette vertical, no bubbles!)

ii. Remove any displaced solution from the other side

g. Place the adaptor in the minifuge, make sure the balance chip is present and spin for 4 minutes.

10. Set up the Experiment on the PGM

a. Select 65 cycles (this is for 100bp reads and will take about 2 hours).

b. Select Autoanalysis and Pre Analysis

c. When the sample has been loaded on the chip and spinning is complete, hit NEXT on the PGM.

i. OK will confirm current setting

ii. Cancel will return you to the Configuration screen if you need to change anything.

iii. As prompted, put the chip into the clamp and close it

1. NO GLOVES

2. Ground yourself on the plate before picking up the chip and putting it in place.

d. Follow the instructions on the screen to begin calibration, which takes ~ 1 minute.

i. Once calibration is complete, check for any leaks around the clamp – if there are none close the lid, if there are any press abort and try reseating the chip. Then press Calibrate.

ii. If the chip passes Calibration press NEXT

1. After 90 seconds the run will begin.

e. The Data is automatically sent to the Server when the run is complete

11. The instrument should be cleaned after every two runs, or if it sits for more than 10 hours.