**Alternative to use of Neutralization Buffer for ES process (OT2 kits)**

*Note: Many of my actual deviations from the official Ion protocol are shown in ‘blue’; alterations that merely consolidate text are not necessarily identified. I still use this protocol with the HiQ Templating & Sequencing kits.*

***10 February 2016***

# Prepare and install the amplification solution

Note: Immediately before preparing amplification rxn, prepare OT2 for the run ( \_\_ Breaking Solution, 150 ul).

**IMPORTANT!**  Start run on Ion OT2 ≤15 minutes after preparing the amplification solution.

1. Prepare reagents per Ion protocol:
	1. Reagent Mix (RT): Bring to room temperature; vortex 30 s; centrifuge ~2 s; inspect for precipitate (this is easier if you remove the yellow strip with a single-edge razor blade).
	2. Enzyme (4oC): Flick tube (upside-down) to ‘mix’; centrifuge ~2 s; put on ice.
	3. ISPs (RT): Place suspension at room temperature.
2. Dilute Library to \_\_\_\_ pM **Serial Dilution**:

**Straight Dilution: \_\_\_** ul Stock library @ \_\_\_\_\_\_\_ pM

\_\_\_ ul Stock library @ \_\_\_\_\_\_\_ pM \_\_\_ ul TVLE.

\_\_\_ ul TVLE [10 mM Tris, 0.05 mM EDTA]. \_\_\_ ul Serial Dil. #1 @ \_\_\_\_\_\_\_ pM

\_\_\_ ul TVLE.

* 1. \_\_\_ Standard: Vortex diluted library (5 s); centrifuge (2 s); put diluted library on ice.
	2. \_\_\_ Optional (e.g., Low-Complexity Libraries): Just before mixing reagents, denature diluted library for 5-10’ at ~96oC; Snap-Cool diluted library by placing tube in ice-water slurry for >2’.
1. To a 2-mL tube (violet cap) with 800 μL of Hi-Q™ Reagent Mix, add the following components in the designated order. Add each component, then pipet amplification solution up and down to mix. (Note: The CS can be replaced with water; however, the CS is a good QC measure of the OT2, even if a genome reference is used. If purely a QC control, the CS volume could likely be ≤5 µl.)
2. 15 µl Nuclease-free water
3. 10 µl Calibration Standard (CS – Orange cap)
4. 50 µl Ion PGM™ Hi Q™ Enzyme Mix (Brown cap)
5. 25 µl Diluted library (not stock library)
6. Add the Ion PGM™ Hi‑Q™ OT2 ISPs:
	1. Vortex ISPs at maximum speed for *1 minute* to resuspend the particles.
	2. Centrifuge ISPs for 2 seconds.
	3. Pipet ISPs up and down to mix.
	4. *Immediately* add ISPs, and then proceed to next step.
7. 900 µl Master-mix (from above)
8. 100 µl Ion PGM™ Hi Q™ ISPs (Black cap)
9. Vortex amplification solution prepared in step 4 at maximum speed (5 s), then centrifuge (2 s). Proceed *immediately* to "Fill and install Ion OneTouch™ Reaction Filter.
10. Pipet amplification solution up-&-down to mix; then, slowly pipet the 1 mL of amplification solution through the sample port.
11. Add 1.7 mL Ion OneTouch™ Reaction Oil through the sample port:
	1. With a new 1000-μL tip, slowly pipet 850 μL of Reaction Oil through the sample port; then... with plunger of pipette still depressed... remove the tip from the sample port.
	2. Attach a new pipette tip; repeat step ‘a’.
12. With sample port on your left, rotate the assembly to your *right* until the Reaction Tube is inverted and the 3 ports of the Reaction Plus Filter face *down*:



1. Install the Reaction Plus filter on the OT2 as per Ion protocol.
2. Start OT2 as per Ion protocol.

# Recover the template-positive Ion PGM™ Hi‑Q™ ISPs

--Note: Immediately before starting this procedure,

* Add **600** ul OneTouch™ Wash Solution to a new 1.5-mL Eppendorf LoBind® Tube for the ISPs; label tube.
* Do Steps A & B of Enrichment prep.
* Ensure heat block is set to 50oC.
1. At the end of the run, follow screen prompts to centrifuge sample... per Ion protocol.
2. *Immediately* after centrifuge has stopped, on instrument display, touch **Open Lid**... per Ion protocol.
3. *Carefully* remove both Ion OneTouch™ Recovery Tubes from the instrument... per Ion protocol.
	* Set ISP tubes in 50°C hot block while processing (to ensure no SDS precipitation exists).
4. Remove excess Ion PGM™ OT2 Recovery Solution from Ion PGM™ Template OT2 ISPs.
	1. Do **not** disturb the pellet of ISPs.
	2. Use a Pipette to remove all but ~100 μL of Recovery Solution (RS) from each Recovery Tube.
	3. Withdraw supernatant from surface; start with a P-1000 (initially sliding tip around entire rim to suck up any white flocculent material) and finish with a P-200 tip on opposite side from pellet.
	4. Do ***not*** store the recovered ISPs in Ion PGM™ Hi‑Q™ ISPs Recovery Solution.
5. Process the ISPs:
	1. With a new tip and using same tip for both tubes, resuspend ISPs in remaining OT2 Recovery Solution (i.e., pipet up and down ~25-50X to disperse pellets). Transfer both suspensions to the LoBind® Tube (with **600** ul Ion OneTouch™ Wash Solution; see “Note” above).
	2. Using a different pipette & tip, add 200 μL of Ion OneTouch™ Wash Solution to the bottom of each Recovery Tube (*to avoid including any flocculent material adhered to the tube sidewalls*).
	3. Using Pipette & tip from Step 5a, pipet to disperse any remaining ISPs in Recovery Tubes.
		1. Transfer suspensions from both tubes to the LoBind® Tube, and cap tube.
		2. Vortex 1.5-ml tube briefly to fully mix.
		3. Note: If desired, sample can be stored at 4oC at this point.
6. Heat the ISPs at 50°C for 2-3 minutes (to ensure no SDS precipitation exists).
7. Centrifuge ISPs for **3** minutes (*my centrifuge timer doesn’t do ½ minutes*) at 15,500 × *g*.

-- Do Steps C & D of “Enrichment Prep” now.

1. With a P-200, reduce the volume to ~70-80 μL of Wash Solution (WS); then restore to 100 μL.
	* 1. Withdraw supernatant from the surface and on the opposite side from pellet;
		2. After measuring the volume of WS in tube, add sufficient WS to bring to 100 μL.
		3. Pipette to resuspend pellet.
2. Take a 2-µl aliquot for Qubit analysis.
	1. Combine 2 µl ISP sample, 19 µl Annealing Buffer, and 1 µl Probes.
	2. Pipet rxn 50-100X to mix; put in thermocycler @ 95oC (2’); 37oC (2’); RT indefinitely.
	3. Clean-up: Add 200 ul QC Wash Solution & vortex; 2’ spin; remove to ~10 ul. Repeat 2X. Add 200 ul WS; pipet 5X & transfer to assay tube for Qubit reading.
3. Begin the Enrichment protocol.

# Enrich the template-positive Ion PGM™ Hi‑Q™ Ion Sphere™ Particles

**Prepare reagents, then fill the 8-well strip (per protocol).**

1. Prepare Melt-Off Solution... per Ion protocol [40 ul freshly-made 1M NaOH (from 10 M stock stored in dark); and, 280 ul TWEEN (from kit)].
2. Wash and resuspend the Dynabeads® MyOne™ Streptavidin C1 Beads... per Ion protocol.
3. Per Ion protocol: fill 8-strip; prepare/run ES module, except **OMIT** steps for Neutralization Solution.
	1. Confirm that a new tip and an opened, **EMPTY** 0.2-mL PCR tube (i.e., **WITHOUT** any Neutralization Solution) are loaded and that the 8-well strip is correctly loaded. Ensure that Well 1 (ISP sample) is the left-most well and that the strip is in far-right position within slot.
	2. Per Ion protocol... pipette beads in Well 2 to resuspend (if needed) and operate the ES module.
	3. IMMEDIATELY after the run, proceed to “**Remove & wash the enriched ISPs**”.

Notes:

1. Ensure that the 0.2-mL PCR tube has >200 μL of solution containing the enriched ISPs. After a successful run, the sample is in ~220 μL of Melt-Off Solution and Ion OneTouch™ Wash Solution.
2. If the tube has <<200 μL of solution, contact LifeTech Technical Support.
3. NO neutralization solution! ... **wash enriched ISPs within 15** **minutes** of the end of the ES run.
4. Prepare a low-bind 1.5 ml tube with 200 ul Wash Solution.

# Remove and SUPER-wash the enriched ISPs

1. 1st Wash of enriched ISPs:
2. Transfer sample to a LoBind® 1.5 ml tube (with 200 ul Wash Solution).
3. Rinse original 0.2-ml tube with 200 ul WS (pipetting up-&-down >25X) transferring contents to the 1.5-ml LoBind® tube.
4. Centrifuge 1.5-ml tube (enriched ISPs), 15,500 × *g* (**2-3** min)
5. Check for Dynabeads® MyOne™ Streptavidin C1 Beads (a brown-tinted pellet); if present:
	1. Remove all but ~50-μL of supernatant from 1.5-ml tube; do not disturb pellet.
	2. Add ~170-μL WS; pipet up-&-down ~25-50X to resuspend ‘brown’ pellet (beads + ISPs).
	3. Place 1.5-mL PCR tube against magnet (e.g., DynaMag™-2 magnet) for 4 min.
	4. Transfer supernatant with enriched ISPs to a NEW 1.5-mL tube without disturbing beads.
	5. Centrifuge 1.5-mL PCR tube with enriched ISPs at 15,500 × g (2-3 min).
6. 2nd wash of ISPs:
	1. Remove all but ~30-50 μL of supernatant from LoBind® tube without disturbing pellet (~ invisible).
	2. Resuspend pellet (pipetting up-&-down ~25-50X), and transfer contents to a **NEW** 0.2-ml LoBind® tube (Using NEW tubes helps eliminate carry-over of enrichment components.).
	3. Quickly rinse 1.5-ml tube with ~170 ul WS (pipetting up-&-down >25X), pooling the contents with the concentrated ISPs in the new 0.2-ml tube.
	4. Centrifuge 0.2-mL PCR tube with enriched ISPs at 15,500 × *g* (**2-3** min)
7. 3rd wash of ISPs:
	1. Remove all but ~10 μL of supernatant from 0.2-ml tube without disturbing pellet.
	2. Quickly resuspend pellet in ~200 ul WS (pipetting up-&-down ~25-50X).
	3. Centrifuge the 0.2-mL PCR tube with enriched ISPs at 15,500 × *g* (**2-3** min).
8. Final preparation:
	1. Remove all but ~10 μL of supernatant from 0.2-ml tube without disturbing pellet.
	2. Quickly resuspend pellet in ~200 ul WS by pipetting up-&-down ~25-50X (no AB when adding TFs.).

\_\_\_ Std. Libraries: Proceed with workflow.

\_\_\_ Low-Complexity libraries: split sample into 3 NEW 0.2-ml tubes (~65 ul each); add ~135 μL

WS to each tube, pipetting >25X; and, continue workflow, processing diluted ISPs in parallel.

**>>> Sequence or store the template-positive ISPs**

**Chip Check**... per Ion protocol. \_\_\_ For Low-Complexity Libraries, perform Chip Check now to minimize time ISPs are at minimal volumes; otherwise, you can wait until the normal point in the official Ion protocol.

# Prepare the template-positive ISPs for sequencing:

-- For initializing PGM, use either 1 M NaOH (7 µl in W2; 35 µl in W1) or 100 mM NaOH (70 µl in W2; 350 µl in W1)

\_\_\_ Low**-Complexity Libraries: Do *NOT* start this**

**step until the PGM is fully initialized and ready for loading chip.**

**Add Control Ion Sphere™ Particles and anneal Sequencing Primer to enriched ISPs**

Note: The ISPs are difficult to see. To avoid aspirating the particles in the following steps, orient the PCR tube the same way each time when centrifuging so that it is easy to know where the pellet has formed, and remove the supernatant from the top down.

1. Vortex Control Ion Sphere™ Particles (~**10-15** s, on high) and centrifuge (~2 s) before taking aliquots.
2. Add Control Ion Sphere™ Particles directly to the entire volume of enriched, template-positive ISPs in a 0.2-mL PCR tube (non-polystyrene):
	1. \_\_\_ Std Libraries: 5.0 ul Seq. Kit TFs (*with 87% loading on a 318 chip, I’ve gotten ~76K TF\_1 & 24K TF\_A reads*).
	2. \_\_\_ Low-Complexity Libraries: ~1.6 ul of TFs per tube; process the 3 tubes in parallel below.
3. Mix thoroughly, pipetting up-&-down ~25-50X.
	* Note: Do not use Annealing Buffer.
4. *Do* ***NOT*** *proceed with this step until PGM initialization is complete, particularly with Low-complexity libraries.* Centrifuge the tube(s) for 2-3 minutes at 15,500 × g.

(Note: The Ion 400bp protocol normally uses a Neutralization Solution in the Enrichment process, and this buffer also provides the necessary salt environment for primer annealing. Thus, that protocol replaces Steps 3 & 4 with: “Mix the contents of the tube by thoroughly pipetting up and down. Centrifuge for 2 minutes at 15,500 × g.”. Here, since we’re avoiding the neutralization buffer, we are using extra WS to perform the buffer exchange. )

Note: In Steps 5-7, it is easiest to use two pipettes so that the tip used in Steps 5b & Step 7 can be left on the pipette.

1. Without disturbing the pellet, prepare sample for annealing the Primer:
	1. \_\_\_ **Std. Library**:
		1. Remove sufficient supernatant such that the remaining volume is slightly <15 μL; visually compare sample tube to two reference tubes (one @ 10 μL and the other @ 15 μL);
		2. measure volume with P20 pipette; save pipette tip for Step 6; and,
		3. using a fresh tip, add Annealing Buffer to bring final volume to 15 μL.
	2. \_\_\_ **Low-Complexity Libraries**:
2. Remove sufficient supernatant such that the remaining volume per tube is ~15 μL; visually compare sample tube to two reference tubes (one @ 10 μL and the other @ 15 μL);
3. measure volume with P20 pipette; save pipette tip for Step 6; and,
4. using a fresh tip, add Annealing Buffer to bring final volume to 25 μL in each tube.
5. Add Sequencing Primer
	1. \_\_\_ Std. Library: Add 12 ul of Sequencing Primer.
	2. \_\_\_ Low-Complexity Libraries: Add 8 μL of Sequencing Primer to each of the 3 tubes.
6. Using the tip saved from Step 5, pipet sample up-&-down (~25-50X) to thoroughly disrupt pellet.
7. Put tube(s) in a thermal cycler, using the heated lid option and these parameters:
	1. \_\_\_ Standard Libraries: 95°C, 2 minutes; 37°C, 2 minutes.
	2. \_\_\_ Low-Complexity Libraries: 95°C, **5** minutes; 37°C, 2 minutes.
8. After cycling,
	1. \_\_\_ Std. Library: the reaction can remain in cycler at RT while you proceed with Chip Check.
	2. \_\_\_ Low-Complexity Libraries: proceed IMMEDIATELY to addition of Sequencing Polymerase.

# Bind Sequencing Polymerase to the ISPs

1. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler.
	1. \_\_\_ Std. Libraries: add 3.0-**3.25 μL** of Sequencing Polymerase to ISPs (~30 μL total final vol.).
	2. \_\_\_ Low-Complexity Libraries: add **1.25-1.5 μL** of Seq Polymerase to ISPs (~32 μL total final vol.).
2. Pipet the sample up-&-down (~50-100X) to mix.
3. Incubate sample at room temperature for 5 min (up to 10’ with Standard Libraries).
	1. While waiting, prep PGM chip for loading (see ‘Load Chip’), if Chip Check is already done.
	2. Keep samples at **Room Temperature – Do NOT put at** 4oC.

Note: In the past, I often stored polymerase-bound samples at 4oC for several hours; however, with recent Low-Complexity libraries, this has resulted in poor loading, apparently due to “clumping” of the ISPs while processing them in a single tube. Splitting such samples (as described above) might help minimize or eliminate this ‘clumping’.

**Chip Check**... per Ion protocol. (*Note: Step has already been done for Low-Complexity Libraries.*)

# Load chip:

**Remove liquid from the chip**

1. Tilt the chip 45 degrees so that the loading port is the lower port.
2. Insert the pipette tip firmly into the loading port and remove as much liquid as possible from the loading port. Discard the liquid.

IMPORTANT! For the next steps, balance centrifuge adapter with a used chip as follows:

* 1. Same chip *type* (e.g., 314, 316, 318) and *orientation* (i.e., Tab IN or OUT).
	2. Balance an inverted chip with another inverted (i.e., upside-down) chip (Tab IN).
	3. Fully load right-side up balance-chips with Annealing Buffer (A.B.).
	4. Mark the used chip to differentiate it from the new chip containing the sample.
1. \_\_ Optional: Vacuum chip, and then blow-dry it (by ‘closing’ off T-valve in vacuum line; *see* [*Home-made Vacuum line for aspirating liquid from Loaded chips.*](http://ioncommunity.lifetechnologies.com/thread/7093)).
2. Place chip **upside-down** in centrifuge adapter bucket and transfer bucket to MiniFuge **with the chip tab pointing IN** (toward center of MiniFuge).
3. Centrifuge (~10 s, from starting centrifuge) to completely empty the chip.
4. Remove chip from bucket, and wipe off any liquid in bucket.

**Load the sample on the chip**

IMPORTANT! When loading liquid into the chip, keep pipette tip at a 90o angle to the chip, press the tip firmly into the circular loading port, and apply gentle pressure between the pipette tip and chip.

***Note: The protocol below is written for 318 chips; it should work fine for 316 chips, but modifications will be required for 314 chips.***

1. Place chip back in centrifuge adapter bucket and hold in air at ~45o tilt with loading port in lower corner. (Alternative: place bucket on a flat, stable surface such as a benchtop, as per Ion’s protocol.)
2. Following polymerase incubation, collect entire sample (~35 μL) into a Rainin® SR-L200F pipette tip, and insert the tip firmly into the loading port of the chip.
	1. \_\_\_ Std. Libraries: first add 5-ul A.B. (*to ensure sufficient volume throughout loading process*) to ISPs.
	2. \_\_\_ Low-Complexity Libraries: no additional A.B. necessary.
3. Dial-down the pipette to gently and slowly deposit the ISPs at a rate of ~1 μL per second. To avoid introducing bubbles into the chip, leave a small amount of sample in the pipette tip (~1 μL).
4. Recapture any displaced liquid from other chip port by dialing-up the pipette with tip in the liquid; retain tip on pipette and lay pipette down such that the tip will remain ‘clean’ for use in later steps.
5. Transfer the chip to the MiniFuge with the chip tab pointing **IN** (toward the center of the MiniFuge), and centrifuge for 30 seconds.

(*Note: Do* ***NOT*** *use the Weighted Buckets... use only the original standard centrifuge adapter buckets.*)

1. Mix sample in chip (retain chip in centrifuge bucket, or remove it as per Ion protocol).
	1. Use pipette tip from Step 4 above; ensure that all liquid in pipette tip is consolidated at bottom of tip. If needed, eject sample into original sample tube, spin tube, and dial sample back into tip.
	2. Tilt the chip 45 degrees so that the loading port is the lower port, and insert the pipette tip (with liquid at end of tip) into the loading port.
	3. Without removing the tip,
		1. First, slowly dial the sample out of the chip until the liquid-front nears the loading port.
		2. Then, normally pipette the sample in-&-out of the chip 3-4X (taking care not to suck the sample entirely out of the chip or to overflow the exit port).
			1. If the chip is not full, add several microliters of Annealing Buffer to the original sample tube, dial it into the pipette tip, and resume mixing.
			2. Watch the liquid line when mixing the sample; if bubbles appear or if liquid becomes isolated in the chip, centrifuge the chip (Tab OUT, ~10-15 s) to move remaining sample towards loading port and resume mixing. *Spin moves bubbles towards outlet port; thus, some liquid may ‘burst’ out of the outlet port upon resumption of mixing.*
		3. Finally, quickly dial sample back into chip until it just begins to well out of the exit port.
2. Centrifuge the chip for 30 seconds with the chip Tab pointing OUT (away from center of MiniFuge).
3. Final Loading procedures:
	1. \_\_\_ Std. Libraries: Repeat MixingCentrifuging steps:
		1. Centrifuging chip (30 s) with Tab IN.
		2. Centrifuging chip (30 s) with Tab OUT.
		3. Centrifuging chip (30 s) with Tab IN.
4. \_\_\_ Low-Complexity Libraries: Swap samples *(note: use a new tip if any ‘clogging’ occurs)*:
	1. Remove Sample #1 from chip, returning it to Sample Tube #1.
	2. Repeat Steps 2-7 with Sample Tube #2
	3. Remove Sample #2 from chip, returning it to Sample Tube #2.
	4. Repeat Steps 2-7 with Sample Tube #3
	5. Remove Sample #3 from chip, returning it to Sample Tube #3.
	6. Repeat Steps 2-7 with Sample Tube #1
	7. Remove Sample #1 from chip, returning it to Sample Tube #1.
	8. Repeat Steps 2-7 with Sample Tube #2
	9. Remove Sample #2 from chip, returning it to Sample Tube #2.
	10. Repeat Steps 2-7 with Sample Tube #3
	11. Remove Sample #3 from chip, returning it to Sample Tube #3.
5. Empty chip: Tilt chip at a 45-degree angle and slowly remove as much liquid as possible from loading port by dialing the pipette. Save the liquid in refrigerator until certain sample is not needed.
6. Perform a quick spin (5-10 s) with the chip tab pointing OUT; remove and pool any additional liquid with the recaptured material from Step 11.
7. Ensure that all liquid is removed from the chip (Do not flush the chip.): *Note: a vacuum line can be made from an obsolete PGM component as described in* [*Home-made Vacuum line for aspirating liquid from Loaded chips.*](http://ioncommunity.lifetechnologies.com/thread/7093)*.* (*NOTE: high vacuum can remove ISPs from near the loading port*);
	1. pipette out majority of sample, quick-spin chip (tab-out) & remove last visible liquid;
	2. \_\_ Optional: VERY lightly blow-dry chip (by briefly, but repeatedly, ‘closing’ off T-valve in vacuum line); and finally,
	3. perform a final 10-s inverted spin (tab-in).

**Standard Libraries tracking chart:**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Cycle #1** | **Cycle #2** | **Cycle #3** | **Cycle #4** | **Cycle #5** | **Final** |
| **Mix in chip** | Initial Loading,Step 3 \_\_\_| |  |  |  |  | Unload, with 10sQuickspin ‘out’ \_\_\_| |
| **Spin Tab** | IN | OUT | IN | OUT | IN | \_\_ Blow-dry (option);\_\_ Inverted spin(10 s) ‘tab-in’ \_\_\_| |
|  | Normal spin (30 s, tab-out)\_\_\_| |

**Low-Complexity Libraries tracking chart:**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Tube 1** | **Tube 2** | **Tube 3** | **Tube 1** | **Tube 2** | **Tube 3** |
| **Load Sample** |  |  |  |  |  |  |
| **Spin Tab** | IN | IN | IN | IN | IN | IN |
| **Mix in chip** |  |  |  |  |  |  |
| **Spin Tab** | OUT | OUT | OUT | OUT | OUT | OUT |
| **Unload,** **with 10 s****Quickspin ‘out’** |  |  |  |  |  | >> Unload, with 10sQuickspin ‘tab-out’\_\_|>> Inverted spin (10 s) ‘tab-in’ \_\_\_|>> Normal spin (30 s), ‘tab-out’ \_\_\_| |

1. When chip loading is complete, press **Next** on the touchscreen and proceed immediately to Select the Planned Run and perform the run.

*Note: I have also tried splitting “****Low-Complexity Samples****” into 6 tubes following enrichment. In that protocol, each tube received ~0.8 ul of TFs and 5 ul Sequencing Primer; further, each tube was loaded onto the chip one time. The protocol worked, but I am not convinced that the results were any better than would have been achieved with a 3-tube split... and the 6-tube split required more effort.*