GS FLX+ Upgrade, Titanium emPCR Quick Guide

Procedure for LVE 2 Cups and SVE 4 Projects Preparations

(March 8, 2012)

1.0 Preparation of the Live and Mock Amplification Mix

1.1 Prepare the Live Amplification Mix in the Amplification Reagents enclosure.

Reagent	LVE 2 Cups	SVE 16 reaction
ddH_2O	2550 µl (was 2740 ul)	1285 µl
emPCR Additive	3000 µl	1440 µl
5x Amplification Mix	1720 µl (was 1560 ul)	760 µl
Amplification Primer	150 µl (was 120 [460] µl)	55 µl (was 220 µl)
Enzyme Mix	400 µl	200 µl
PPiase	10 µl	8 µl
Total Volume	7830 µl	3748 µl

Vortex for 5 sec., store at +2°C to +8°C until ready for use.

1.2 In a separate tube, dilute the 5x Mock Amplification Mix to its working concentration, as follows:

a. Place 2 **ml** (**1 ml** for SVE) of 5x Mock Amplification Mix in a 15 ml Falcon tube.
b. Add 8 **ml** (**4 ml** for SVE) of Molecular Biology Grade Water.
c. Cap, vortex to mix, and store at +2°C to +8°C until ready for use.

2.0 DNA Library Capture

2.1 Washing the Capture Beads

(1) Dilute 10x Capture Bead Wash Buffer to 1x (1 ml Buffer + 9 ml ddH2O).
(2) Vortex two tubes (one tube for SVE) of the DNA Capture Beads. (For SVE only: Aliquot capture beads into four 1.7 ml tubes, 320 µl for each tube.)

Note: Each tube contain **35 x 10^6** beads in 1170 µl. (For SVE the single tube from kit contains 34.8 **x 10^6** beads in 1280 µl.)

(3) Pellet the beads in a bench top minifuge.
   a. spin for 10 seconds,
   b. rotate the tube 180°, and
   c. spin again for 10 seconds.
(4) Remove and discard as much of the supernatant as possible without disturbing the beads pellet, then:
   a. Add **1 ml** of 1x Capture Bead Wash Buffer to each tube of beads, and
   b. Vortex for 5 seconds to resuspend the beads.
(5) Repeat step 3 and 4.
(6) Pellet the DNA Capture Beads in the minifuge, and remove and discard as much of the supernatant as possible without disturbing the beads.

2.2 DNA Library Capture

To each tube of washed DNA Capture Beads, add the correct volume of the DNA library to provide optimal amplification. To determine this volume, you need to:

(1) Have determined the concentration of the library and use 0.8x as the optimal ratio of DNA molecules per beads to use; These determinations are done for each library, at the end of the library preparation procedure (see the *GS FLX Titanium General Library Preparation Method Manual*).

(2) For LVE: Know that the number of DNA Capture Beads **per tube** is **35,000,000 In each of two separate 1170 ul containing tubes.** (for SVE **each tube** is **9.600,000 after dividing between 4 separate tubes.)**

(3) Use the following equation:

$$\mu l \text{ of library needed per tube} = \frac{0.8 \text{ molecules per bead desired} \times \text{number of beads per tube}}{\text{library concentration (in molecules/}\mu l)}$$

(4) Vortex the tubes for 5 seconds to mix their contents.

(5) Prepare the DNA Capture Beads with library DNA mixes for individual emulsion reactions:

For LVE:

a. Transfer each captured library mix to a clean 15 ml conical tube; save the pipette tips.

b. Add **1 ml** of Live Amp Mix to each of the microcentrifuge tubes, rinse using the saved pipette tips, and add to the 15 ml tube.

c. Add another **2.75 ml** of Live Amp Mix to each 15 ml tube (total about 3 ml); Store at +2°C to +8°C until you are ready to add them to the cups of emulsion.

Note: It is best to amplify the beads immediately after adding the DNA templates. However, if the beads will not be used immediately, they should be stored at +2°C to +8°C until needed, at which time the protocol can resume at the following step. DNA libraries should be aliquot for single use. Any unused library should be discarded and not refrozen.

For SVE:

a. Aliquot of captured library mixes into 4-0.5ml tubes for each tube (~ 40 µl per tube). To each aliquot of captured library mixes, add **215 µl** of Live Amplification Mix (Prepared in section 1.1).

b. Store at +2°C to +8°C until you are ready to add them to the tubes of emulsion (section 3.0, step 4).

3.0 Emulsification

(1) Titanium: Pre-mix the Emulsion Oil at **28 Hz for 2 minutes (25 Hz** for SVE).

(2) Add **5 ml** (**290 µl** for SVE) of 1x Mock Amplification Mix to each cup (each tube for SVE) of Emulsion Oil for LVE. Invert the cups or tube racks 2-3 times

to mix.

(3) Place the cups (tubes) into the TissueLyser. Shake in the TissueLyser for **28 Hz (25 Hz for SVE)** for **5 min.**

(4) Add the captured library preparations (from section 2). Vortex and pour the content from a 15 ml tube of captured library into each cup of pre-emulsion. Invert the cups 2-3 times to mix.
(For SVE, pipette up and down and add the whole content (about **255 µl**) from a 0.5ml tube of captured library to each tube of pre-emulsion. Invert the tube racks 2-3 times to mix.)

(5) Shake in the TissueLyser for **12 Hz (15 Hz** for SVE) for **5 min.**

3.1 Dispensing the Emulsions

After emulsification, dispense the emPCR amplification mixes into 96-well thermocycler plates.
For LVE: Each cup of emulsion will fill approximately 180 wells at 100 µl per well. A full LVE emPCR kit will usually fit into four plates.
For SVE: Each tube of emulsion will fill approximately 10 wells. A full SVE emPCR kit will usually fit into two plates.
Dislodge any air bubbles that may be present in the well.

4.0 Amplification Reaction

(1) Bring the plates containing the emulsified amplification reactions and place them in a thermocycler

(2) Set up and launch the following amplification program: (Titanium emPCR)
   a. 1x (4 minutes at 94°C)
   b. 50x (30 seconds at 94°C, 10 minutes at 60 °C)
   c. 10°C on hold until halted by user

After completion of the amplification program (about 6 h run time); remove the plates of amplified material from the thermocycler.
Do not freeze the DNA beads: You can leave the amplification reactions at 10°C for up to 16 hours before further processing the samples.

5.0 Bead Recovery

5.1 Bead Resuspension/Pooling

(1) Emulsion breakage: Check all wells for emulsion breakage (*i.e.* a droplet of clear material at the bottom of the well). If the emulsion in any well appears broken, discard the entire well and do not recover the beads from it.

(2) Prepare the following reagents: Prepare 1x Enhancing Fluid TW and 1x Annealing Buffer TW from their concentrates,
   a. Add **187.5 ml** of Molecular Biology Grade Water to the **62.5 ml** of 4x Enhancing Fluid TW stock (in its 250 ml container). Swirl to mix and keep on ice.
   b. Add **72 ml** of Molecular Biology Grade Water to the **8 ml** of 10 x Annealing Buffer TW stock (in its 250 ml container). Shake vigorously and keep on ice. This will be used later.

5.2 Emulsion Breaking using centrifugation

(1) With 12 channel pipette, add ~100ul isopropanol to the first 96 wells for one projects emulsion reaction wells. (48 wells for one project)
(2) Use a syringe to transfer the emulsion-isopropanol mix from same project into a 50ml corning tube (for LVE: put max 96 wells emulsion into one corning tube).
(3) Refill the wells with ~200ul isopropanol to wash the wells.
(4) Transfer the mix into the same corning tube (Cap type: Flat top; Corning Costar No. 430921, Fisher Sci. Cat. No.05-538-67).
(5) Add isopropanol into the corning tube to volume ~40ml (30 ml for SVE).
(6) Repeat steps (1)-(5) for the remaining projects.
(7) Mix the contents in Corning tubes by hand.
(8) Centrifuge the corning tubes at **2600rpm** in room temperature for **5** minutes in the Beckman Table Top Centrifuge.
(9) Carefully pour the supernatant into a waste container (it is better to leave some supernatant, *e.g.* ~5 ml, than to lose beads), and rinse the bead pellets **two times** by add isopropanol to **40 ml** (30 ml for SVE) in each tube and vortex, by centrifugation as above.
(10) Rinse the bead pellets once more, with **35 ml (25 ml for SVE)** of 1x Enhancing Fluid TW. Pellet with centrifugation same as above. The bead pellet will be softer after this wash, so pour the supernatant SLOWLY. Also, leave about 2 ml of 1x Enhancing Fluid TW, *i.e.* stop pouring when the white bead pellet starts coming off from the bottom of the tube.
(11) Transfer the DNA bead suspension into two 1.7 ml tubes for each emulsion cup processed [4 - 1.7 ml tubes for a whole LVE kit] [4 - 1.7 ml tubes for SVE]. Centrifuge the 1.7 ml tubes and remove the supernatant one or more times as required.
(12) Rinse each of the 50 ml collection tube with **600 µl** of 1x Enhancing Fluid TW, and add this rinse to the 1.7 ml tubes. Centrifuge the 1.7 ml tubes and remove the supernatant as required.
(13) Wash beads **twice** with **1 ml** of 1x Enhancing Fluid TW for each tube.
(14) Pellet the beads in a bench top minifuge, as follows:
    a. spin for 10 seconds,
    b. rotate the tube 180°, and
    c. spin again for 10 seconds.
    d. Remove and discard the supernatants.

6.0 DNA Library Bead Enrichment

6.1 Preparation for Indirect Enrichment

Turn on the heating dry-block or water bath and set it to 65°C. If necessary,

(1) Prepare a stock of Melt Solution by mixing **125 µl** of NaOH (10 N) in **9.875 ml** of Molecular Biology Grade Water. This solution will be used in this section and in section 6.4.(1) below.
(2) Prepare the ssDNA beads by melting the dsDNA amplification products (only one strand is attached the beads). Do the following **twice**:

a. Add **1 ml** of Melt Solution per tube of beads. **Vortex**. **Incubate** for **2 min**.
b. Pellet the beads in a bench top minifuge, as above
c. Remove and discard the supernatants.

Risk of DNA degradation: Never leave the DNA beads in Melt Solution for more than 10 minutes.

(3) Wash the bead pellets **twice** by centrifugation as above, with **1 ml** **(500 µl for SVE)** of **1x Annealing Buffer** per tube each time.

(4) **Add 45 µl** (30 µl for SVE) 1x Annealing Buffer TW with beads, **Add 25 µl** per tube (24 µl per tube for SVE) **Enrichment Primer**. **Vortex**.

(5) Anneal the Enrichment Primer to the bead-bound ssDNA by placing the tubes in a heat block at **65°C for 5 min**, and then promptly cooling on **ice for 2 min**.

(6) **Add 800 µl** **(500 µl for SVE)** of 1x Enhancing Fluid TW. **Vortex**.

(7) Pellet the beads by centrifugation as above, and remove the supernatants.

(8) Wash the bead pellets **twice** more by centrifugation, with **1 ml** **(500 ul for SVE)** of **1x Enhancing Fluid TW** per tube each time.

(9) After removing the supernatant, resuspend each tube of beads pellet in **800 µl** of 1x Enhancing Fluid TW. **Vortex**.

6.2 Preparation of the Enrichment Beads

(1) Vortex the tube of Enrichment Beads for **1 minute.**
(2) Place tube on MPC to pellet beads.
(3) Remove and discard all supernatant.
(4) Wash the pelleted beads **twice** with **1 ml** of 1x Enhancing Fluid TW, using the MPC. To ensure proper washes, take the tube out of the MPC and vortex each time.
(5) After removing the supernatant, remove the tube from the MPC, and add **320 µl** of 1x Enhancing Fluid TW into each tube and Vortex.

6.3 Enrichment of the DNA-Carrying Beads

(1) Add **80 µl** washed Enrichment beads into each tube of amplified DNA beads.
(2) **Vortex** to mix completely the DNA Beads and the Enrichment Beads.
(3) **Rotate on a LabQuake** tube roller at room temperature for **5 minutes**.
(4) Place the tubes in the MPC, and wait 3-5 minutes to pellet the paramagnetic Enrichment Beads against the side of the tubes. Cap the tubes and **invert** the magnet several times. The supernatants will remain milky at this point.
(5) Carefully remove and discard the supernatants from each tube using a 1000 µl pipette, taking care not to draw off any pelleted Enrichment Beads.
(6) Wash the beads with **1 ml** **(500 µl for SVE)** of 1x Enhancing Fluid TW per tube as described below, as many times as necessary until there are no visible beads remaining in the supernatants (usually 6-10 washes for LVE; **3-6 washes** for SVE).
a. Add **1 ml** (500 **µl for SVE)** of 1x Enhancing Fluid TW per tube.
b. Remove the tubes from the MPC and vortex them.
c. Place the tubes back into the MPC to pellet the beads.
d. Carefully remove and discard the supernatant from each tube.

Optionally, collect the supernatants at each wash for troubleshooting (pool with the collected supernatants of step 5), and/or to spin and monitor when washes are complete.

6.4 Collection of the Enriched DNA Beads

(1) Remove the tubes from the MPC and resuspend each bead pellet in **700 µl** of Melt Solution.
(2) Vortex for 5 seconds, and put the tubes back into the MPC to pellet the Enrichment Beads.
(3) Transfer the **supernatants** containing enriched DNA beads from two tubes (one cup) (One tube for SVE) to a separate 1.7 ml microfuge tube.
(4) For LVE only: Spin the beads for 10 seconds, rotate the tube, and spin again. Discard the supernatant.
(5) Repeat steps 1-4 for better DNA bead recovery, pooling together the two melts from each pair of tubes (from each single tube for SVE), and discard the tubes of spent Enrichment Beads.
(6) Spin the beads for 10 seconds, rotate the tube, and spin again and then discard the supernatant.
(7) Wash the bead pellets **three times** with **1 ml (500 µl for SVE)** of 1x Annealing Buffer XT by centrifugation to completely neutralize the Melt Solution.
(8) Resuspend each of the final beads pellets in **200 µl** (120 **µl for SVE)** of 1xAnnealing Buffer XT.

7.0 Sequencing Primer Annealing

(1) Add **50 µl** per tube [**24 µl** per tube (6 µl per SVE emulsion reaction equivalent)] of **Sequencing Primer**. **Vortex**.
(2) Place the tubes in a heat block at **65°C for 5 min**, and then promptly on **ice for 2 min**. Add **800 µl (500 µl for SVE)** of 1x Annealing Buffer XT, pellet the beads as before, and remove the supernatant.
(3) Wash each of the bead pellets **twice** with **1 ml (500 µl for SVE)** of 1x Annealing Buffer XT.
(4) Resuspend each of the final bead pellets by adding to **1 ml** of Annealing Buffer XT.
(5) To determine the % bead enrichment, do the following:
   a. Count a 5 µl aliquot of the beads in 2 ml buffer with a particle counter.
   b. Calculate the % bead enrichment using the following equation:

$$\% \text{ Bead Enrichment} = \frac{\text{Number of enriched beads}}{\text{Total input beads*}} \times 100$$

***35 x 10^6** beads per emulsion cup **(2.4 x 10^6** beads per emulsion reaction for SVE) included in this sample.

   c. Percent enrichment values between 5 and 15% are indicative of libraries Yielding good sequencing results.
(6) Store the beads at +2°C to +8°C. The stored beads will remain sequenceable for at least 1 month.