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454
SEQUENCING

TCB No. 011-2009

June 2009

Technical Bulletin

Genome Sequencer FLX System

emPCR Kit and Protocol Updates for the GS FLX Titanium Series

Summary

This bulletin describes changes and improvements to the GS FLX Titanium series emPCR Kits including the use of new emPCR Additive components (pre-amplification). These updated methods are intended for use with both General and Paired End libraries and enable the user to maximize sequencing quality and performance of the GS FLX System.



Note: This bulletin is intended to serve as an addendum to the existing *GS FLX Titanium emPCR Method Manual (April 2009)*. This document alone does not provide sufficient instructions to use the GS FLX Titanium LV & SV emPCR Kits. Relevant sections of the Method Manual are referenced in this document and should be modified or replaced as directed herein. This update pertains to the GS FLX Titanium series only – not Standard series reagents or methods.

Introduction

This document describes several improvements to the GS FLX Titanium LV & SV emPCR Kits and associated methods. These changes represent the latest update to the emulsion PCR components of the 454 Sequencing System. These new methods, including the use of the emPCR Additive, are intended for general use and should be employed as described in all projects using GS FLX Titanium series reagents.



Caution: The improved GS Titanium emPCR Reagents SV (Lib-L), mat. no. 05233631001, from kit lots 93726160 and subsequent, are only intended for use with GS FLX emPCR Kit Titanium Emulsion Oil SV, mat. no. 05233640001, from kit lot 93692660 or kit lot 93734320 and subsequent (i.e. higher lot numbers).

Changes to the Method Manual

Modifications to procedures outlined in the *GS FLX Titanium emPCR Method Manual (April 2009)* are described below. Protocol changes are separated by sections identical in decimal number and name to those in the Method Manual. Significant changes to steps, including those describing the use of new reagents (i.e. emPCR Additive) or methods are highlighted in **red text**.

2.2.2 GS FLX Titanium emPCR Kits

Table 2-1 on p. 15 of the Method Manual is revised as follows to reflect the addition of the emPCR Additive to the Titanium emPCR Reagents box (one tube for SV Kit, two tubes for LV Kit):

| Box Name | Temperature | | Kit component | Kit Part | Quantity |
|---------------------------------|-------------|----------------|---------------------------------|----------|--|
| | Ship | Store | | | |
| Titanium Bead Recovery Reagents | RT | +2°C to +8°C | 10X Annealing Buffer TW | B | 8 ml |
| | | | 4X Enhancing Fluid TW | B | 62.5 ml |
| | | | Enrichment Beads | B | 320 µl |
| Titanium emPCR Reagents | Dry ice | -15°C to -25°C | 5X Amplification Mix | A | 2 x 800 µl ^a 1 x 800 µl ^b |
| | | | 5X Mock Amplification Mix | A | 2 x 1.0 ml ^a 1 x 1.0 ml ^b |
| | | | emPCR Enzyme Mix | A | 2 x 200 µl ^a 1 x 200 µl ^b |
| | | | PPiase | A | 10 µl |
| | | | 10X Capture Bead Wash Buffer TW | A | 1.2 ml |
| | | | Amplification Primer | A | 460 µl |
| | | | DNA Capture Beads | A | 2 x 1.17 ml ^a 1 x 1.28 ml ^b |
| | | | emPCR Additive | A | 2 x 1.5 ml^a 1 x 1.5 ml^b |
| | | | Enrichment Primer | B | 100 µl |
| | | | Sequencing Primer | B | 100 µl |
| Titanium Emulsion Oil | RT | RT | Emulsion Oil | A | 2 x 10 ml ^a 16 x 0.6 ml ^b |

Table 2-1: Composition of the GS FLX Titanium emPCR Kits

Each kit is shipped in 3 packages, according to the shipping/storage temperature requirements of the components. RT: Room Temperature (+15 to +25°C). The differences between the two kit sizes are identified as follows: ^a**GS FLX Titanium LV emPCR Kit (Lib-L)**; and ^b**GS FLX Titanium SV emPCR Kit (Lib-L)**.

3.1 Preparation of the Live and Mock Amplification Mixes

Steps 1-3 (pp. 21-22) should be modified as follows:

1. In the Controlled Room, open the emPCR Reagents box. Prepare the reagents for use, as follows:
 - a. Allow the frozen kit components to thaw fully, except for the enzyme components.



Leave the enzyme tubes (emPCR Enzyme Mix and PPIase) at -15°C to -25°C at this time.

- b. After they thaw, vortex the reagents for 5 seconds.
 - c. If a precipitate is observed after thawing the tube of emPCR Additive, vortex the tube well to dissolve it.



If precipitation remains in the emPCR Additive after vortexing, heat the reagent to 55°C in a heat block for up to five minutes to aid in dissolving. If residual precipitate remains, briefly centrifuge the tube and use the supernatant in the preparation of the Live Amplification Mix.

2. Spin all the kit components that are in microcentrifuge tubes (including enzymes) in a bench top mini centrifuge for 10 seconds. Then:
 - a. Return the enzymes to -15°C to -25°C.
 - b. Transfer the other kit reagents to the Amplification Reagents enclosure.
3. In the Amplification Reagents enclosure, prepare the Live Amplification Mix for the size and number of emulsion reactions being made, according to Table 3-1 A (for General libraries) and B (for Paired End libraries). Use a tube of the appropriate size for the amount of Live Amplification Mix you are preparing.



For multiple emulsions, prepare the total amount of Live Amplification Mix you will need in a single tube, even if your emulsions will be of different DNA libraries. The mix will be pipetted into the individual emulsion cups or tubes later (section 3.2, step 11).

A (General libraries):

| Reagent | LV Kit | | SV Kit | | |
|-----------------------|----------------|----------------|---------------|----------------|----------------|
| | 1 Cup | 2 Cups | 4 Tubes | 8 Tubes | 16 Tubes |
| Mol. Bio. Grade Water | 1200 µl | 2400 µl | 280 µl | 560 µl | 1120 µl |
| emPCR Additive | 1500 µl | 3000 µl | 360 µl | 720 µl | 1440 µl |
| 5X Amplification Mix | 780 µl | 1560 µl | 190 µl | 380 µl | 760 µl |
| Amplification Primer | 230 µl | 460 µl | 55 µl | 110 µl | 220 µl |
| emPCR Enzyme Mix | 200 µl | 400 µl | 50 µl | 100 µl | 200 µl |
| PPIase | 5 µl | 10 µl | 2 µl | 4 µl | 8 µl |
| Total: | 3915 µl | 7830 µl | 937 µl | 1874 µl | 3748 µl |

Table 3-1 A: Preparation of the Live Amplification Mix for General libraries

B (Paired End libraries):

| Reagent | LV Kit | | SV Kit | | |
|-----------------------|--------------------|--------------------|-------------------|--------------------|--------------------|
| | 1 Cup | 2 Cups | 4 Tubes | 8 Tubes | 16 Tubes |
| Mol. Bio. Grade Water | 1370 μl | 2740 μl | 320 μl | 640 μl | 1285 μl |
| emPCR Additive | 1500 μl | 3000 μl | 360 μl | 720 μl | 1440 μl |
| 5X Amplification Mix | 780 μl | 1560 μl | 190 μl | 380 μl | 760 μl |
| Amplification Primer | 60 μl | 120 μl | 15 μl | 30 μl | 55 μl |
| emPCR Enzyme Mix | 200 μl | 400 μl | 50 μl | 100 μl | 200 μl |
| PPiase | 5 μl | 10 μl | 2 μl | 4 μl | 8 μl |
| Total: | 3915 μl | 7830 μl | 937 μl | 1874 μl | 3748 μl |

Table 3-1 B: Preparation of the Live Amplification Mix for Paired End libraries

Users will typically process a full kit to prepare sample DNA beads for a full PicoTiterPlate device, so the columns for 2 cups and 16 tubes will be used the most often (highlighted). The Live Amplification Mix prepared will then be pipetted into individual emulsion reactions (cups or tubes; see section 3.2, step 11). Volumes can be scaled up or down, for different size experiments (fractional sequencing Runs or multiple sequencing Runs). Volumes for 1 cup and for 4 or 8 tubes are given for convenience; in such cases, the rest of the emPCR kit can then be saved for a subsequent experiment.

- Vortex the Live Amplification Mix for 5 seconds, and store it at +2°C to +8°C (in the Controlled Room refrigerator or on ice) until ready for use (section 3.2, step 11).
- In a separate tube, dilute the 5X Mock Amplification Mix to its working concentration, as follows:
 - Place 2 ml (or 1 ml for SVE) of 5X Mock Amplification Mix in a 15 ml Falcon tube.
 - Add 8 ml (or 4 ml for SVE) of Molecular Biology Grade Water.
 - Cap, vortex to mix, and store at +2°C to +8°C (in the Controlled Room refrigerator or on ice) until ready for use (section 3.3, step 4).

If you did not prepare the entire kit worth of Live Amplification Mix, you can either:

- Prepare a proportional amount of Mock Amplification Mix and re-freeze the rest of the concentrated stock for future use by the expiration date of the kit; or
- Prepare the whole amount and store the left over amount at +2°C to +8°C for future use within 4 weeks or by the expiration date of the kit, whichever occurs first.

3.2 DNA Library Capture

Modifications have been made to the Capture Bead component of the GS FLX Titanium LV and SV emPCR Kits for all kit lots beginning with LV kit lot 93717360 and SV kit lot 93726160 and subsequent (i.e. higher lot numbers). This change requires a modification to the emPCR process. For the above-listed lots, it will be necessary to add more library than before to the capture beads (step 9 in section 3.2 of the *GS FLX Titanium emPCR Method Manual* or step 6 section 2 of the *GS FLX Titanium emPCR Quick Guide*).

- If the amount of DNA is determined using the Emulsion Titration Assay or the Full Sequencing Titration Assay, then the points of the titration curve should be modified to accommodate the new higher DNA amount requirements. This affects:
 - section 3.10.3, step 1 of the *GS FLX Titanium General Library Preparation Method Manual*: create a 2×10^6 molecules/ μl dilution of the library instead of the 1×10^6 dilution, by mixing 2 μl of the 1×10^8 stock in 98 μl of TE Buffer.
 - section 3.10.3, step 2 of the *GS FLX Titanium General Library Preparation Method Manual* and section 3.2, step 9 of the *GS FLX Titanium emPCR Method Manual* (and section 2, step 6 of

the *GS FLX Titanium emPCR Quick Guide*): increase the amount of DNA per tube (in cpb*) per the Table below. Note that the volumes to be pipetted do not change; only the working concentration.

| Tube | Instead Of | Replace With |
|------|--|--|
| 1 | 0.5 cpb – 1.2 µl of a 1×10^6 solution | 1 cpb – 1.2 µl of a 2×10^6 solution |
| 2 | 1 cpb – 2.4 µl of a 1×10^6 solution | 2 cpb – 2.4 µl of a 2×10^6 solution |
| 3 | 2 cpb – 4.8 µl of a 1×10^6 solution | 4 cpb – 4.8 µl of a 2×10^6 solution |
| 4 | 4 cpb – 9.6 µl of a 1×10^6 solution | 8 cpb – 9.6 µl of a 2×10^6 solution |

*cpb = copies per bead = molecules per bead

- If a fixed cpb has been established by local laboratory SOP, or a calculated cpb is used (as determined by some other means), then replace these values with approximately **three (3) times more library** than would have been previously used. *For example:* if the local laboratory's SOP calls for 1 cpb for a certain sample type, then 3 cpb should be used instead, with the kit lots listed above.

3.5 Bead Recovery

There is no change required for processing of Small Volume Emulsions (SVE). For Large Volume Emulsions (LVE), replace Section 3.5.1.3: Bead Washes and Recovery for Large Volume Emulsions (LVE) as follows (**Steps 3-8** below replace Steps 4-5 (p. 33) in the original Method Manual; Steps 9-13 below are identical to Steps 6-10 in the Method Manual but are provided for in the interest of completeness):

- Mix the contents of the two (or more) 50 ml collection tubes by transferring their contents (in pairs) back and forth four times or until the bead suspensions are of about the same density.
- Add isopropanol to final volume of **40 ml** in each tube. Vortex.
- Pellet the beads in a centrifuge at 930 x g for 5 min** (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750). Carefully pour the supernatant into a waste container (use a pipette to remove remaining supernatant, if necessary).
- Add 10 ml of 1X Enhancing Fluid TW** and vortex well to resuspend. If vortexing is not sufficient to resuspend completely, use a glass rod or a spatula to break bead aggregates.



Note: It is important to fully suspend the bead suspension **before** adding isopropanol in the next step to ensure complete mixing and to prevent clumping.

- Add isopropanol up to a total volume of 40 ml** and vortex well to resuspend. Pellet the beads in a centrifuge at **930 x g for 5 min** (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750). Carefully pour the supernatant into a waste container (use a pipette to remove remaining supernatant, if necessary).
- Add 35 ml of isopropanol** and vortex well to resuspend. Pellet the beads in a centrifuge at **930 x g for 5 min** (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750). Carefully pour the supernatant into a waste container (use a pipette to remove remaining supernatant, if necessary).
- Add 35 ml of ethanol** and vortex well to resuspend. Pellet the beads in a centrifuge at **930 x g for 5 min** (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750). Carefully pour the supernatant into a waste container (use a pipette to remove remaining supernatant, if necessary).

8. Add 30 ml of 1X Enhancing Fluid TW and vortex well to resuspend. Pellet the beads in a centrifuge at 930 x g for 5 min (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750). Carefully pour the supernatant into a waste container. The bead pellet will be softer after this wash, so pour the supernatant SLOWLY. Also, leave behind about 2 ml of 1X Enhancing Fluid TW, i.e. stop pouring when the white bead pellet starts coming off from the tube bottom.
9. Transfer the DNA bead suspension to two 1.7 ml tubes for each emulsion cup processed [4 tubes for a whole GS FLX Titanium LV emPCR Kit (Lib-L)].
 - Depending on the amount of 1X Enhancing Fluid TW left in the 50 ml tubes, you may have to centrifuge the 1.7 ml tubes (see step 11, below) and remove the supernatant one or more times to collect all the beads in the correct number of 1.7 ml tubes.
10. Rinse each of the 50 ml collection tubes with 600 µl of 1X Enhancing Fluid TW, and add this rinse to the 1.7 ml tubes.
 - Again, you may have to centrifuge the 1.7 ml tubes and remove the supernatant to collect all the beads in the correct number of 1.7 ml tubes.
11. Pellet the beads in a bench top minifuge (the minifuge has only one speed), as follows:
 - a) spin for 10 seconds,
 - b) rotate the tube 180°, and
 - c) spin again for 10 seconds.
12. Remove the supernatant and rinse twice with 1 ml of 1X Enhancing Fluid TW.
 - Make sure to resuspend the bead pellets completely each time, to ensure proper rinsing of the beads.
13. Resuspend the bead pellets in a final 1 ml of 1X Enhancing Fluid TW.



Note: If you are following the LVE procedure, skip section 3.5.2 entirely.

3.7 Sequencing Primer Annealing

Due to the improvements to amplification efficiency resulting from the use of the emPCR Additive, the enrichment percentage range that leads to the best sequencing quality has broadened. Experience to date suggests that enrichment yield up to 20% may be acceptable for good quality sequencing results.

5.1 Materials Required but not Provided

| Reagents/Consumables | Stock Conc. | Quantity Required | Source | Ref. Number |
|----------------------|-------------|-------------------|---------------|-------------|
| Ethanol | 100% | 70ml | Many possible | N/A |