GS FLX Titanium emPCR Method Manual

October 2008

Preface

About this Manual

This manual describes the method to clonally amplify the DNA fragments from a properly prepared DNA library, for sequencing in the Genome Sequencer System. This method is currently limited to non-MID "General" (*e.g.* Shotgun) libraries (see Note, below), and uses one of two available emPCR kits from the Genome Sequencer FLX Titanium series. The two kits differ in the size of the preparation they produce, *i.e.* the number of amplified DNA beads of a given sample:

- The Large Volume Emulsions (LVE) prepared with the GS FLX Titanium **LV** emPCR Kit (Lib-L) generate amplified DNA beads in quantity sufficient to fill the large (30 × 60 mm) loading regions of a PicoTiterPlate device, for applications when the greatest number of reads of a sample is required.
- The Small Volume Emulsions (SVE) prepared with the GS FLX Titanium **SV** emPCR Kit (Lib-L) generate amplified DNA beads in quantities appropriate to fill the medium (14 × 43 mm), the Medium/Small (M/S; 5.4 × 60 mm), or the small (2 × 53 mm) loading regions, based on the amount of sequencing information required for any particular sample.

Where the procedure diverges, a color code specifies the correct details for each kit (see below). In all cases, the emPCR amplification process results in an immobilized library of clonally amplified DNA fragments suitable for sequencing with the Genome Sequencer System (using a GS FLX Titanium series sequencing kit).

The Genome Sequencer System for DNA sequencing was developed by 454 Life Sciences, a Roche company.

Important Note: October 2008 marks the first release of the new GS FLX Titanium series chemistry for the Genome Sequencer FLX System. For the time being, the system supports only the non-MID "General" (*e.g.* Shotgun) sequencing applications under the GS FLX Titanium chemistry. For Paired End or Amplicon sequencing, or for the preparation and sequencing of MID libraries of any type, users must continue to use the GS FLX standard series kits and procedures (last updated in December 2007).

Note, however, that samples prepared using kits and procedures from the GS FLX Titanium series are still sequenced on the same Genome Sequencer FLX Instrument. Also, the Genome Sequencer FLX Software version 2.0, associated with the October 2008 release, can process datasets generated with any of the Genome Sequencer's chemistries (GS 20, GS FLX standard, and GS FLX Titanium). Indeed, the software version 2.0 can co-process reads produced on any combination of Genome Sequencer System chemistries (and even "Sanger reads") within a given analysis, such as in an Assembly, a Mapping, or an Amplicon Variant Analysis Project.

The Genome Sequencer FLX Titanium series manuals are easily identified by their new cover graphics and distinctive tri-color stripes (reflecting the GS FLX Titanium kits packaging). All the methods, protocols and applications supported on the GS FLX standard chemistry will be enabled on the GS FLX Titanium chemistry in the near future.

Incompatible chemistries: The two supported chemistries of the Genome Sequencer System (GS FLX standard and GS FLX Titanium) are completely incompatible with each other. For example, standard libraries cannot be amplified using GS FLX Titanium series emPCR kits, and libraries prepared/amplified with GS FLX Titanium series kits cannot be sequenced on the standard PicoTiterPlate devices, or vice-versa. **It is crucially important that kits and procedures belonging to a single chemistry platform be used throughout the preparation, amplification, and sequencing of a DNA sample.**

The kits and procedures described in this document must be used only to amplify non-MID General libraries prepared according to the *GS FLX Titanium General Library Preparation Method Manual*. All other library types must be amplified using either the GS FLX emPCR Kit I, II, or III, according to the *GS FLX emPCR Method Manual* (December 2007).

In this manual, the phrase "Genome Sequencer System" refers to whole system for DNA sequencing developed by 454 Life Sciences Corp., including the Genome Sequencer Instrument, all the kits for the preparation, amplification and sequencing of a DNA sample, the methods to use the kits as described in the Manuals and Guides, and the software provided to process and analyze the data from sequencing Runs. Likewise, "Genome Sequencer FLX System" refers to a Genome Sequencer System based on the Genome Sequencer FLX Instrument (as opposed to the Genome Sequencer 20 Instrument, which is now retired). Two versions of the Genome Sequencer FLX System have been released: the GS FLX standard series, last updated in December 2007, and the GS FLX Titanium series. 454 Life Sciences Corporation is a Roche company.

Safety

Make sure to follow the precautionary statements presented in this manual. Such statements and other items of special interest are highlighted with the following icons:

Symbol	Heading	Description
	Warning	Indicates the possibility of severe or fatal injury to the user or other persons, or damage to a system component, if the precautions or instructions are not observed.
	Caution	Highlights information that is critical for optimal performance of the system. May also indicate that loss of data or the generation of invalid data could occur if the precautions or instructions are not observed.
	Information Note	Identifies items of general interest and additional information about the topic or procedure being described.
▶▶▶		Table continued on next page.
■		End of table.

Assumptions

Sample ready

This manual describes a method for the clonal amplification of a suitably prepared DNA library, in preparation for a sequencing Run on the Genome Sequencer Instrument. Therefore, the emPCR amplification procedures assume that the user has previously prepared a library of quantitated, "adapted" DNA fragments (DNA library) using the appropriate Genome Sequencer System method and kit, as specified in section 2.2.1 of this manual. Please refer to the *GS FLX Titanium General Library Preparation Method Manual* for more details on this kit and procedure.

Trained personnel

Trained personnel: This manual assumes that the person carrying out the procedures described herein is trained in proper and safe laboratory techniques, and in the correct handling of the kit components. Throughout the documentation of the Genome Sequencer System, the words "user" and "you" refer to properly trained individuals. If you have any questions, please contact your Roche Representative for information about user training.

Color Code

The procedure described in this manual can be carried out on two scales, supported by two separate kits. Wherever the emPCR amplification procedure differs between kit types, kit-specific instructions are shown in a color-coded font, based on the table below.

The procedure for this kit	… is typed in this color
GS FLX Titanium **LV** emPCR Kit (Lib-L)	Regular black type
GS FLX Titanium **SV** emPCR Kit (Lib-L)	Orange type if different from LV

Mostly, orange type will identify reagent volumes specific to the use of the small volume emulsions. However, one section of the procedure, the bead recovery step (section 3.5), has a completely separate set of instructions for the two kits. In that case, the instructions for the LV kit (in black type, section 3.5.1) should be skipped by users of the SV kit; and the instructions for the SV kit appear in orange type, below (section 3.5.2).

1. Introduction to the emPCR Amplification Method

The Genome Sequencer System provides a unique technology for sequencing DNA samples in an efficient, massively parallel, and cost-effective manner. The system provides protocols and reagents for the integrated sequencing process. This includes a step to amplify a library of DNA template fragments from a single, functionally clonal, bead-bound copy, to tens of millions of copies per bead. The bead-bound amplified library can then be sequenced with the Genome Sequencer System. This manual describes the method to carry out such a library amplification, using either the GS FLX Titanium LV emPCR Kit (Lib-L) or the GS FLX Titanium SV emPCR Kit (Lib-L).

This unique, emulsion-based clonal amplification process (emPCR) eliminates the need for biological cloning of the template fragments. While the emPCR amplification process is performed batch-wise on the whole library, it ensures functional clonality by physically separating the DNA-carrying beads in an emulsion during the *in vitro* amplification.

The starting material for the emPCR amplification process is a library of quantitated DNA fragments flanked with proper amplification and sequencing adaptors. For the time being, only non-MID "General" (*e.g.* Shotgun) libraries prepared using the GS FLX Titanium General Library Preparation Kit, in accordance with the *GS FLX Titanium General Library Preparation Method Manual*, can be amplified using the GS FLX Titanium series emPCR kits.

For the preparation of Paired End or Amplicon libraries, or of MID libraries of any type, users **MUST** continue to use the standard Genome Sequencer FLX kits, manuals, and procedures (last updated in December 2007).

The GS FLX Titanium chemistry offers the possibility to carry out the emPCR amplification process on two scales, supported by two similar but separate kits:

- **Large Volume Emulsion Preparation (LVE)**: This protocol uses the GS FLX Titanium LV emPCR Kit (Lib-L). This protocol is especially intended for maximizing the number of reads obtained from a single sample, during a single run. It is carried out in emulsion **cups** and is used to prepare samples for loading into **Large** (30 × 60 mm) regions of a PicoTiterPlate device.
- **Small Volume Emulsion Preparation (SVE)**: This protocol uses the GS FLX Titanium SV emPCR Kit (Lib-L). Procedures for one to four emulsion **tubes** allow for the preparation of samples at appropriate scales for loading into **Medium** (14 × 43 mm), **M/S** (5.4 × 60 mm), or **Small** (2 × 53 mm) regions of a PicoTiterPlate device, according to the amount of sequencing information required for the sample.

The emulsion-based clonal amplification process (emPCR) comprises 7 main steps, as shown in Figure 1–1 and briefly described below. The full procedures for each step are provided in section 3 of this manual.

The Figure illustrates the steps that use Part A of the GS FLX Titanium emPCR Kit (any type) and are carried out in the "Controlled room"; and those that use Part B of the kit and are carried out in the "Amplicon room". This physical segregation is fundamental to the success of the process, since it minimizes the risk of stray amplicons contaminating the amplification reaction. For further detail, see section 2.1 of this manual, or the *Genome Sequencer System Site Preparation Guide*. However, the Bead Recovery (step 5), the DNA Library Bead Enrichment (step 6), and the Sequencing Primer Annealing (step 7) may also be carried out in the General Laboratory, for convenience.

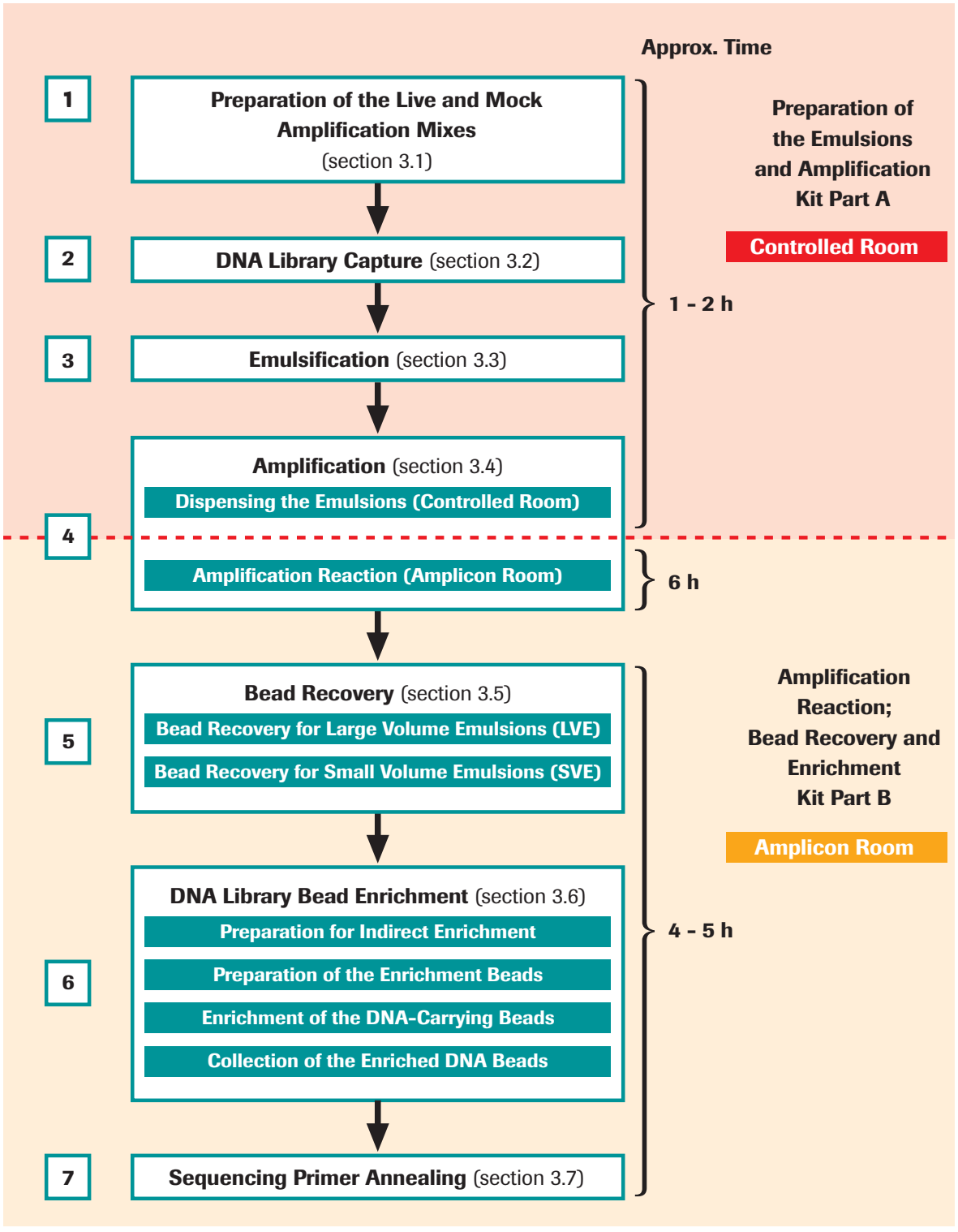
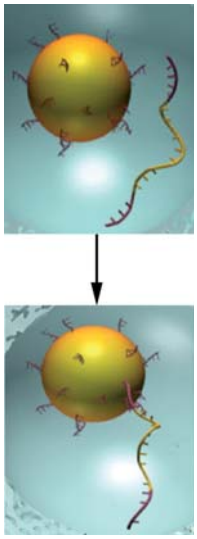


Figure 1–1: The seven-step workflow of the emulsion-based clonal amplification procedure (emPCR)

The full procedure for each step is given in the sections of this manual indicated in parentheses.

1 **Preparation of the Live and Mock Amplification Mixes**: Prepare the amount of live amplification mix required for the experiment. This amount is determined by the size (LVE in cups or SVE in tubes) and number of emulsion reactions to prepare, as described in section 2.3.

For the complete live amplification mix preparation procedure, see section 3.1 of this manual.

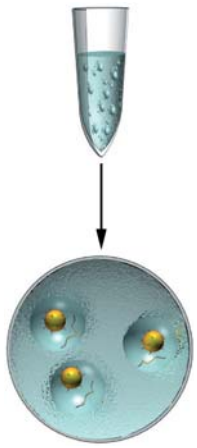


2 **DNA Library Capture:** The fragments that make up the DNA library are immobilized onto DNA Capture Beads, to allow for their segregation in the emulsion. The goal is to achieve one *effective* molecule (a functional clone) of DNA per bead; and one bead per aqueous microreactor (micelle), insulated from other beads by the surrounding oil. It is the segregation of each bead into its own microreactor (with amplification reagents) that will maintain clonality during the amplification step.

The capture process involves hybridization of Adaptor "B", which was attached to the 3'-end of each template molecule during library preparation, to complementary oligonucleotides that are covalently attached to the DNA Capture Beads.

- DNA library capture does not formally occur at this step: the DNA templates are only added to the beads at this point, and physical capture (annealing to the bead-bound oligonucleotides) occurs during the amplification reaction.
- Not every DNA molecule present on the beads will actually get amplified. Therefore, the optimal amount of DNA for an emPCR amplification reaction, the amount that will allow clonal amplification, is the amount that will result in a single molecule of DNA *being amplified* per bead (*i.e.*, a functional clone). This optimal amount of DNA is determined by way of a three-tiered set of options described in the *GS FLX Titanium General Library Preparation Method Manual*.

For the complete DNA library capture procedure, see section 3.2 of this manual.

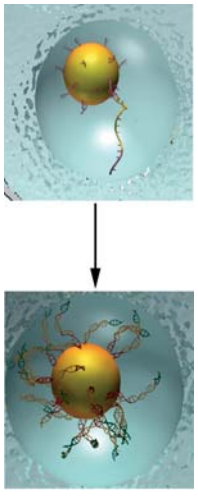


3 **Emulsification:** The captured DNA library is resuspended in the amplification mix and oil, and emulsified to form a water-in-oil mixture. This step involves vigorous mechanical shaking under tightly controlled conditions. The products of the emulsification step are aqueous phase "microreactors" 50 to 100 µm in diameter, each containing all the components of the amplification mix and no more than a single bead.

This step marks the main difference between the two size preparations:

- Large Volume emulsions are made in the **cups** of Emulsion Oil provided in the GS FLX Titanium **LV** emPCR Kit (Lib-L); these are used to prepare samples for loading onto large regions (30 × 60 mm) of a PicoTiterPlate device (one cup per region).
- Small Volume emulsions, on the other hand, are made in the **tubes** of Emulsion Oil provided in the GS FLX Titanium **SV** emPCR Kit (Lib-L); these are used to prepare samples for loading onto Medium (14 × 43 mm; 4 emulsion tubes/region), M/S (5.4 × 60 mm; 2 tubes/region), or Small (2 × 53 mm; 1 tube/region) regions of a PicoTiterPlate device.

For the complete emulsification procedure, see section 3.3 of this manual.

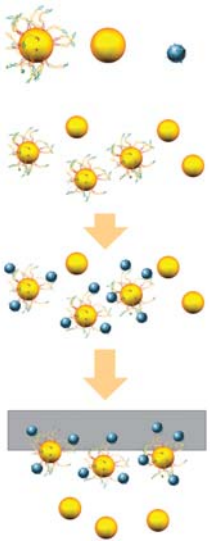


4 **Amplification:** The emulsified beads are subjected to PCR to clonally amplify each template DNA molecule. The DNA templates are hybridized to bead-bound oligonucleotide primers, the capture primers (see step 2, above). These capture primers double as PCR primers, anchoring the newly synthesized, complementary strands to the beads. As the PCR reaction progresses, these bead-bound, complementary strands direct the synthesis of more first-strand moieties, which hybridize to an excess of bead-bound capture primers. Also, the second, soluble amplification primer is biotinylated; this allows for enrichment of the beads carrying amplified DNA later in the procedure (see step 6, below). After amplification, typical immobilized template copy number ranges from 10 to 50 × 10^6 copies per bead.

For the complete amplification procedure, see section 3.4 of this manual.

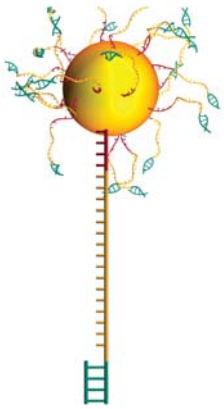
5 **Bead Recovery:** After the amplification step, the emulsion is broken chemically, and the beads carrying the amplified DNA library (double-stranded at this point) are recovered and washed. Here, separate procedures are used for the two sizes of preparation: a high-throughput vacuum-driven procedure for the LVE procedure, and a syringe-and-filter method for SVE.

For the complete bead recovery procedure, see section 3.5 of this manual.



6 **DNA Library Bead Enrichment:** The procedure above generates a certain proportion of beads that carry no amplified DNA (null beads), or little amplified DNA, either because they did not capture a molecule of template in the beginning or because the DNA template did not amplify properly. To reduce the percentage of beads without or with too little template, the sixth step of the procedure enriches the total bead population for amplified DNA-carrying beads. This enrichment step involves the hybridization of the biotinylated Enrichment Primer (from the Kit) to the Adaptor "A" of each amplified template (to which it is complementary), and its binding to streptavidin-coated magnetic beads. The bound beads (carrying amplified DNA) can then be separated from the null and poorly amplified beads with a magnetic particle collector. The DNA library beads are then separated from the magnetic beads by melting the amplification products away from the Enrichment Primer, leaving a population of bead-bound single-stranded template DNA fragments: the immobilized and amplified DNA library.

For the complete DNA library bead enrichment procedure, see section 3.6 of this manual.



7 **Sequencing Primer Annealing:** The final step in the emPCR amplification process is the annealing of the sequencing primer to the immobilized, amplified DNA templates.

For the complete sequencing primer annealing procedure, see section 3.7 of this manual.

The entire amplification process takes just a few hours and can be performed by a single individual in a suitably equipped laboratory. The final product of the emPCR amplification process is a library of clonally amplified, bead-immobilized, single-stranded DNA fragments. The DNA library beads are then ready for loading onto a PicoTiterPlate device and sequencing on the Genome Sequencer Instrument with the GS FLX Titanium Sequencing Kit XLR70. (See the *GS FLX Titanium Sequencing Method Manual* for details.)

2. Before You Begin

This section describes the main prerequisites for the emulsion-based clonal amplification of a DNA library (emPCR amplification), as described in section 3 of this manual. Before starting an emPCR amplification experiment, make sure that all the requirements listed in this section are met. The section is divided into three parts:

- Section 2.1: Physical Installations
- Section 2.2: What You Should Have Before Starting
- Section 2.3: Kit Selection; Determining the Size and Number of Reactions to Prepare

2.1 Physical Installations

As with all PCR-based procedures, contamination of the amplification mix with extraneous DNA species can yield poor results. To minimize the risk of contamination, the GS FLX Titanium series emPCR Kits and procedures are divided into two parts:

- Use Part A reagents to prepare the amplification mixes in a segregated, amplicon-free environment.
- Use Part B reagents for the amplification and post-amplification steps, performed in a separate and segregated space.

Thus, for successful emPCR amplification, your laboratory must comprise two distinct, dedicated preparation areas:

- **Controlled Room:** A designated space for performing all steps of the procedure up to amplification.
- **Amplicon Room:** A dedicated room for performing all remaining steps of the procedure, starting with amplification.
  - For convenience (*e.g.* access to a centrifuge that can handle 50 ml conical tubes, for LVE bead recovery), the latter part of the procedure starting with bead recovery (section 3.5) may be carried out in a General Laboratory, rather than in the Amplicon Room.

(See the *Genome Sequencer System Site Preparation Guide* for details on how these rooms should be set up.)

2.2 What You Should Have Before Starting

2.2.1 Sample

Before beginning the emPCR amplification process, you must have already prepared (and quantitated, as appropriate) a DNA library for the GS FLX Titanium chemistry. For the time being, GS FLX Titanium series emPCR kits can be used to amplify only non-MID "General" (*e.g.* Shotgun) libraries prepared using the GS FLX Titanium General Library Preparation Kit, in accordance with the GS FLX Titanium General Library Preparation Method Manual.

This type of library consists of single-stranded DNA fragments averaging between 500 and 900 nt in length. Each DNA fragment is flanked with two adaptors containing the amplification and sequencing primer sequences, as well as a "sequencing key" (used for base calling): Adaptor A at the 5' end and Adaptor B at the 3' end. As part of the preparation procedure, you must have determined the optimal quantity of library needed for clonal amplification in emPCR amplification reaction.

This library preparation procedure also supports initial DNA samples that are inherently of low molecular weight. If the starting material was low molecular weight DNA, the average fragment length of the prepared library may be considerably shorter than indicated above.

2.2.2 GS FLX Titanium emPCR Kits

2.2.2.1 GS FLX Titanium emPCR Kit Components

There are 2 types of GS FLX Titanium emPCR Kits available. Both serve the same function, and differ only in the size of the emulsion reactions that they support and hence, the number of beads per sample that they can produce:

- The GS FLX Titanium **LV** emPCR Kit (Lib-L) provides the Emulsion Oil in two large **cups**. It is used to make the large number of library beads required to load the Large regions (30 × 60 mm) of a PicoTiterPlate device, for maximum throughput on a DNA sample.
- GS FLX Titanium **SV** emPCR Kit (Lib-L) provides the Emulsion Oil in sixteen smaller **tubes**. It is used to make the smaller number of library beads required to load the Medium (14 × 43 mm; 4 tubes/region), M/S (5.4 × 60 mm; 2 tubes/region), or the Small (2 × 53 mm; 1 tube/region) region of a PicoTiterPlate device, for maximum throughput on a DNA sample, according to the amount of sequencing information required for the sample. Also, because the total throughput of the SV kit is somewhat less than that of the LV kit, some of the reagents are provided in lesser quantity, as appropriate.

Each kit can be used to prepare DNA library beads in quantity sufficient to load an entire PicoTiterPlate device, whatever loading gasket is selected.

- The GS FLX Titanium chemistry offers separate procedures for emulsion breaking and bead recovery, matching the two emulsion sizes. However, since multiple emulsions of a given sample can be pooled and the beads recovered with the same breaking apparatus, these are not provided in the emPCR kits, but sold separately. See section 2.2.3 for a description of the two GS FLX Titanium emPCR Breaking Kits.
- The (Lib-L) suffix given to the two GS FLX Titanium emPCR kits described above indicates that they are designed to amplify libraries prepared with ligated Adaptors. In a future release of the GS FLX Titanium chemistry kits, this will include not only General libraries but also Paired End Libraries, both prepared with or without Multiplex Identifiers (MIDs). In addition, two additional emPCR kits will be released which will bear a (Lib-A) suffix, and will be used to amplify libraries prepared with annealed Adaptors, such as Amplicon libraries, prepared with or without MIDs.

Each kit is packaged and shipped in a set of 3 boxes (plus a separate Breaking Kit; see section 2.2.3), according to the shipping and storage requirements of the components they contain. Table 2–1 lists the contents (with volume of each component) of each box.

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

Two components of the Bead Recovery Reagents box (the 10× Annealing Buffer TW and the 4× Enhancing Fluid TW) as well as one from the emPCR Reagents box (the 10× Capture Bead Wash Buffer TW) need to be diluted to their working concentration before use (see section 5.2). After dilution, make sure to mark the bottles (special label in the back) to indicate that you did so. (Two other concentrated reagents, the 5× Amplification Mix and the 5× Mock Amplification Mix, are reconstituted to their working concentration as part of the procedure.)

In addition to this physical, 3-way packaging, the kits are divided *functionally* into two parts:

- Part A contains the reagents required to prepare the Emulsion and Amplification reactions, which must be carried out in the Controlled Room (amplicon-free environment);
- Part B contains the reagents used in the recovery and enrichment of the DNA-carrying beads from the emulsion (after amplification), which are done in a segregated Amplicon Room.
  - For convenience (*e.g.* access to a centrifuge that can handle 50 ml conical tubes, for LVE bead recovery), the latter part of the procedure starting with bead recovery (section 3.5) may be carried out in a General Laboratory, rather than in the Amplicon Room.

Reagent contamination risk: To avoid contamination of the pre-amplification reagents, open the boxes that contain Part A reagents (emPCR Reagents box and Emulsion Oil) only in the Controlled Room, as described in section 3 of this manual. The two Part B reagents shipped in the emPCR Reagents box, the Enrichment and Sequencing Primers, can be taken to the Amplicon Room along with the sample, when that part of the procedure is reached.

2.2.2.2 GS emPCR Kit Storage

To ensure the longest shelf life for your GS FLX Titanium emPCR Kits, store them following the guidelines provided in Table 2–2. Once diluted, they can be stored at +2°C to +8°C for up to 4 weeks.

Box Name	Shipping Conditions	Long-term Storage	Short-term Storage (≤ 4 wk)
Bead Recovery Reagents	Room Temp.	+2°C to +8°C*	+2°C to +8°C*
emPCR Reagents	Dry ice	-15°C to -25°C	---
Emulsion Oil	Room Temp.	Room Temp.	---

Table 2–2: Guidelines for the long-term and short-term storage of the various components of the GS FLX Titanium emPCR Kits

Long-term is up to the expiration date printed on the label, and short-term is up to 4 weeks. Room Temperature storage should be between +15°C and +25°C. *The "Bead Recovery Reagents" have a shelf life of 4 weeks at +2°C to +8°C, after dilution.

2.2.3 GS FLX Titanium emPCR Breaking Kits

Each of the two emPCR amplification reaction formats supported by the GS FLX Titanium chemistry, the so-called Large Volume Emulsion (LVE) and Small Volume Emulsion (SVE) formats, has a corresponding emulsion breaking process that is especially tailored for it, and requires special supplies. The GS FLX Titanium series of kits provide the necessary materials for this.

However, there is a variable relationship between the number of emulsions prepared and the number of breaking set ups needed, because when multiple emulsions are made of the same DNA sample, they can be pooled and broken together. This is common and can occur both with LVE, *e.g.* if the user wants to load both regions of a PicoTiterPlate device with the same library; and with SVE, *e.g.* pool 4 emulsion tubes to load a Medium region or 2 tubes for a M/S region. For this reason, the emulsion breaking supplies are not included in the emPCR kits, but are provided separately.

2.2.3.1 GS FLX Titanium emPCR Breaking Kits, LV, 12 pcs

This kit contains the materials for 12 LVE emulsion breaking setups. Each setup can be used to break the emulsions and collect the beads from any number of LVE cups, as long as they contain the same library. Its usage requires two standard 50 ml conical tubes for each emulsion cup processed, and a vacuum source with moisture trap. See section 3.5.1 for a description of how it is used.

Box Name	Temperature		Kit component	Quantity
	Ship	Store		
GS FLX Titanium emPCR Breaking Kits, LV, 12 pcs	RT	RT	GS FLX Titanium emPCR Breaking Kit, LV	12

The vacuum system can be located either in the Amplicon room or in a general laboratory (see section 2.1). The minimum requirement of the vacuum system is 25 SCFH, or 0.42 CFM (about 11.9 l/min).

2.2.3.2 GS FLX Titanium emPCR Filters, SV, 64 pcs

This kit contains filters for 64 SVE emulsion breaking filters. Each filter can be used to break the emulsions and collect the beads from up to 4 SVE tubes (as long as they contain the same library), and requires a standard 10 ml Luer-Lock syringe. See section 3.5.2 for a description of how these filters are used.

Box Name	Temperature		Kit component	Quantity
	Ship	Store		
GS FLX Titanium emPCR Filters, SV, 64 pcs	RT	RT	GS FLX Titanium emPCR Filters, SV	64

2.2.4 Materials Required but Not Provided

Some pieces of equipment or standard laboratory supplies, as well as some third party reagents, are not provided but are required to carry out the emPCR amplification procedures described in this manual. These items are listed in section 5.1. Make sure that all necessary items are available prior to beginning an experiment.

2.3 Kit Selection; Determining the Size and Number of Reactions to Prepare

The size and number of emulsion amplification reactions needed for a library depends on the throughput requirements in the subsequent sequencing reaction(s). To determine which of the GS FLX Titanium emPCR kits best suits your needs and how many emulsion reactions to make, proceed as follows:

1. Use Table 2–3, columns 1 to 5, to identify the size and number of bead loading regions best suited to yield the sequencing throughput required for your DNA library samples. As a guide, column 4 gives the expected throughput of a full sequencing Run carried out with the Run script that yields the longest reads available (350 to 450 nt), with all regions loaded. In certain cases, *e.g.* if you do not intend to use the longest possible reads, the throughput in reads/region (column 5) may be more relevant than the bases/region.
   - For example, if the experiment aims to sequence a 10 Mbp genome at 20-fold average depth, a total throughput of 200 Mbp is required; this can usually be obtained in a single Large region of a PicoTiterPlate device. An 80 Mbp genome at 20-fold depth would require 1.6 Gbp of total throughput; such a project would require about 8 Large regions, or 4 full sequencing Runs with the Large regions bead loading gasket. But resequencing a 4 Mbp bacterial genome at 8-fold average depth (32 Mbp total throughput requirement) would normally fit comfortably in a single M/S region.
2. Determine the appropriate kit and number of emulsion reactions to prepare for your library, using Table 2–3, columns 5 and 6.
   - The examples above would require respectively 1 cup (half an LV Kit), 8 cups (4 full LV kits; 4 sequencing Runs), and 2 tubes (one eighth of an SV Kit).
   - If your library will not use all the regions of the PicoTiterPlate device, you can use the other regions to load another library and sequence them together, in the same Run.

Region Size	Regions per PTP Device	Bases/Region (Mbp)	Bases per Full PTP Device (Mbp)	Reads/Region ($\times 10^3$)	Kit	Cups/Tubes per Region
Large	2	180–280	360–560	450–650	LV	1 cup
Medium	4	60–110	240–440	160–250	SV	4 tubes
M/S	8	30–55	240–440	80–120	SV	2 tubes
Small	16	10–20	160–320	25–40	SV	1 tube

Table 2–3: Selecting the proper GS FLX Titanium emPCR Kit, and determining the number of emulsion reactions to prepare for a sequencing experiment

This table lists the typical sequencing throughput for each loading region configuration (in Mbp and in reads) for an "extra long reads" sequencing Run carried out using each region size and the GS FLX Titanium Sequencing Kit XLR70. Compare these numbers with the throughput requirements of your experiment (in bases or reads, as appropriate), and determine the appropriate region size and number of regions. Then, choose an appropriate GS FLX Titanium emPCR Kit and determine the number of emulsion reactions to prepare. Note that in all cases, one full emPCR kit always fills one full PicoTiterPlate device. M/S: Medium/Small.

- The Mbp throughput values given in Table 2–3 are typical for good "Extra Long Read" (200 cycles) sequencing Runs performed on good quality Shotgun libraries. However, many factors influence the actual throughput of a Run, such as the nature (and quality) of the library being sequenced. For example, DNA libraries made from low molecular weight DNA will obviously generate shorter reads, and therefore less total throughput (in Mbp) than a properly prepared Shotgun library. Make sure to consider these factors when determining the throughput requirement and the throughput expected in an experiment. In some cases, expressing the throughput in number of reads may be more appropriate than in number of bases.
- It is most efficient and economical to carry out a sequencing Run with all the PicoTiterPlate regions loaded with samples. In certain circumstances, *e.g.* if few small but urgent samples need to be sequenced, users may nonetheless choose to run them on a partially loaded PicoTiterPlate device. When preparing for such a fractional sequencing Run, only part of the GS FLX Titanium emPCR kit is used and the rest can be kept for use at a later date; however, even a fractional sequencing Run will consume a complete GS FLX Titanium Sequencing Kit.
- If the emPCR amplification procedure does not produce the number of beads suggested for loading into the region size you intend to use for your sequencing Run (see the *GS FLX Titanium Sequecing Method Manual*), the beads obtained may still be worth sequencing.

3. Procedure

This section provides the detailed procedure for the emulsion-based clonal amplification (emPCR) of one DNA library sample. See section 2.3, above, to determine the size and number of emulsion reactions needed to generate enough DNA library beads for your sample. There are a few procedural differences related to the use of Large Volume Emulsions (LVE) or Small Volume Emulsions (SVE); special instructions for SVE are highlighted in orange type.

The emPCR amplification process has 7 main steps (see Figure 1–1), which are performed in two separate, dedicated lab areas -- the Controlled Room and the Amplicon Room:

- Section 3.1: Preparation of the Live and Mock Amplification Mixes
- Section 3.2: DNA Library Capture
- Section 3.3: Emulsification
- Section 3.4: Amplification
  - Dispensing the Emulsions

Controlled Room

  - Amplification Reaction
- Section 3.5: Bead Recovery
- Section 3.6: DNA Library Bead Enrichment
- Section 3.7: Sequencing Primer Annealing

Amplicon Room

- **Contamination risks:** If the amplification reaction mix is contaminated with stray amplicons or other extraneous DNA prior to amplification, these contaminants may become a significant portion of the final sequence data. To reduce the risk of contaminating the samples, perform all steps prior to the actual amplification reaction (section 3.4.2) in the Controlled Room (see section 2.1), within UV-treated PCR enclosures or hoods that have been thoroughly pre-cleaned *e.g.* with NucleoClean Wipes.
  - Follow standard good laboratory practices to avoid contamination.
  - Reagents and equipment from the Amplicon Room should never be shared with, or moved into, the Controlled Room areas used to prepare the amplification reactions.
  - Personnel who have been in Amplicon Room areas should exercise good laboratory practices and undergo decontamination before entering Controlled Room areas.
- **Correct pipeting:** All tubes in the GS emPCR kits contain excess reagent. DO NOT simply mix the complete contents of the reagents tubes even if you are preparing all 2 (LV) or 16 (SV) reactions, as this would result in incorrect final reagent concentrations and poor amplification.
  - **Mini-centrifuge:** The mini-centrifuge used in this procedure must develop a relative centrifugal force of 2000 × *g*. Make sure that the one you will be using meets this specification.

3.1 Preparation of the Live and Mock Amplification Mixes

Contamination risk: Optimally, the Controlled room should contain two PCR enclosures, or hoods. The Live Amplification Mix must be prepared in the Amplification Reagents enclosure, within the Controlled room. To prevent the contamination of your samples, if a separate Emulsion enclosure is available, never introduce any DNA in the Amplification Reagents enclosure.

Immediately before use, prepare the amount of Live and Mock Amplification Mixes required to process the number of emulsions needed for your experiment (see section 2.3):

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 during thawing.
   b. After they thaw, vortex the reagents for 5 seconds.
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. Use a tube of the appropriate size for the amount of Live Amplification Mix you are preparing.
   If making 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).

Reagent	LV Kit		SV Kit		
	1 Cup	2 Cups	4 Tubes	8 Tubes	16 Tubes
Mol. Bio. Grade Water	2700 µl	5400 µl	640 µl	1280 µl	2560 µl
5× 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**	**937 µl**	**1874 µl**	**3748 µl**

Table 3–1: Preparation of the Live Amplification Mix

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 pipeted 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.

4. 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).

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

   a. Place **2 ml** (or **1 ml** for SVE) of 5× Mock Amplification Mix in a 15 ml Falcon tube.

   b. Add **8 ml** (or **4 ml** for SVE) of Molecular Biology Grade Water.

   c. 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

Contamination risk: To reduce the risk of reaction contamination, the steps of section 3.2 through section 3.4.1 (inclusive) **must** be conducted in the Controlled Room, in the UV-treated **Emulsion** enclosure (*NOT* the **Amplification Reagents** enclosure!). Thoroughly wipe all surfaces of the Emulsion enclosure with Nucleo-Clean Wipes before use.

1. Prepare 1× Capture Bead Wash Buffer TW by mixing **1 ml** of 10× Capture Bead Wash Buffer TW with **9 ml** of Molecular Biology Grade Water, as described in section 5.2.1.

2. Vortex the two tubes (one tube for SVE) of DNA Capture Beads.
   - These beads will immobilize your amplified DNA library.
     - If you are not preparing a whole kit worth of beads (*i.e.*, you are preparing a fractional sequencing Run), you can aliquot out the amount of DNA Capture Beads you need, and re-freeze the rest for future use. You will then also need to scale other steps accordingly. Note that:
     - Each tube of DNA Capture Beads in the GS FLX Titanium LV emPCR Kit (Lib-L) contains 35 × 10^6 beads in 1170 µl; use one tube of DNA Capture Beads from this kit directly for each cup of emulsion to be processed;
     - The single tube of DNA Capture Beads in the GS FLX Titanium SV emPCR Kit (Lib-L) contains 38.4 × 10^6 beads in 1280 µl; for each emulsion tube to be processed, transfer **80 µl** of DNA Capture Beads suspension from the SV emPCR Kit to a fresh 1.7 ml tube.

       Aliquoting the DNA Capture Beads for SVE: This aliquoting is important because the kit contains an overfill of beads, to guarantee a minimum. Processing too many beads would skew the enrichment calculations and may destabilize the emulsions.

   DNA Capture Beads not interchangeable: The tubes of DNA Capture Beads of the GS FLX Titanium LV emPCR Kit (Lib-L) and the GS FLX Titanium SV emPCR Kit (Lib-L) do not contain the same number of beads and are not interchangeable. Make sure to always use the correct kit components.

3. 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.

4. Remove and discard as much of the supernatant as possible without disturbing the bead pellet, then:
   a. Add **1 ml** of 1× Capture Bead Wash Buffer to each tube of beads, and
   b. vortex for 5 seconds to resuspend the beads.

5. Repeat steps 3 and 4.

6. Pellet the DNA Capture Beads in the minifuge, and remove and discard as much of the supernatant as possible, as above.

7. For SVE only, distribute the washed DNA Capture Beads into single emulsion aliquot size (typically, 16 tubes, for the preparation of a full PicoTiterPlate device with a Medium, M/S or Small regions gasket). Do the following:
   a. Resuspend the pellet of DNA Capture Beads in **640 µl** (or **40 µl** times the number of emulsion tubes you are preparing; see step 1, above) of 1× Capture Bead Wash Buffer.
   b. Distribute evenly between the appropriate number of 1.7 ml (or 0.2 ml) tubes (usually 16 tubes; 40 µl per tube).
   c. Pellet the beads, and remove and discard the supernatant, as before.

8. Thaw an aliquot of the DNA library to be amplified, in the previously UV-irradiated Emulsion enclosure. The library should have been prepared and quantitated according to the *GS FLX Titanium General Library Preparation Method Manual*.

9 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:

a. have determined the concentration of the library and the optimal number of DNA molecules per bead 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*).

b. know that the number of DNA Capture Beads **per tube** is as follows:

I. 35×10^6 for LVE (as provided in the kit)
II. 2.4×10^6 for SVE (after aliquoting, per step 7)

c. use the following equation:

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

d. if necessary, prepare a dilution of the library such that the volume to be added will be:

I. between 1 µl and 100 µl for LVE, and
II. between 1 µl and 10 µl for SVE

If you are carrying out a library titration, which uses the SVE procedure, the dilution (1×10^6 molecules/µl) and volume of diluted library to use in each tube are directly prescribed by the titration assay procedure:

a. Tube 1: 1.2 µl of diluted DNA library (= 0.5 molecule/bead)
b. Tube 2: 2.4 µl of diluted DNA library (= 1 molecule/bead)
c. Tube 3: 4.8 µl of diluted DNA library (= 2 molecules/bead)
d. Tube 4: 9.6 µl of diluted DNA library (= 4 molecules/bead)

See the *GS FLX Titanium General Library Preparation Method Manual* for details.

10 Vortex the tubes for 5 seconds to mix their contents.

11 Prepare the DNA Capture Beads with library DNA mixes for individual emulsion reactions.

a. **For LVE only**, process the samples as follows:

I. Transfer each captured library mix to a clean 15 ml conical tube.
   ▶ The bead suspensions will be quite thick; save the pipette tips used to transfer them (for each separate sample, if applicable), for the tube rinse in the next step.
II. Add **1 ml** of Live Amplification Mix to the microcentrifuge tubes that contained the captured library and, using the pipette tip saved in the previous step (for each separate sample, if applicable), rinse the microcentrifuge tubes and transfer the additional beads thus collected to the 15 ml tubes.
III. Add another **2.75 ml** of Live Amplification Mix to each 15 ml tube, for a total volume of about **3.75 ml**.
IV. Store at +2°C to +8°C (in the Controlled Room refrigerator or on ice) until you are ready to add them to the cups of emulsion (section 3.3, step 8).

b. **For SVE**, process the samples as follows:

I. To each aliquot of captured library mixes, add **215 µl** of Live Amplification Mix.
II. Store at +2°C to +8°C (in the Controlled Room refrigerator or on ice) until you are ready to add them to the tubes of emulsion (section 3.3, step 8).

▶ 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 aliquoted for single use. Any unused library should be discarded and not refrozen.

3.3 Emulsification

- **Contamination risk**: Avoid contaminating the DNA-carrying beads, amplification mixes, and emulsion oil with any extraneous DNA. Wherever indicated below, the procedures (particularly where they involve mixing or dispensing the amplification reaction mix) should be carried out in the Controlled Room Emulsion enclosure.
- **Emulsion oil**: The emulsion oil is subject to phase separation. To assure reproducible emulsions, it is IMPERATIVE that you pre-mix the Emulsion Oil before use (steps 1 and 2, below).
- **Tube position in the TissueLyser**: The TissueLyser (or TissueLyser II) homogenizer allows 16 tube reactions to be emulsified at the same time. **Be aware** that while the homogenizer has a capacity of 48 tubes, **only the outermost row of each tube rack** (when the racks are mounted into the TissueLyser) receives the adequate range of motion to ensure complete emulsification. Emulsions that lead to inconsistent sequencing results have been observed when the innermost or center rows were used for emulsification. (For LVE, the TissueLyser has a capacity of 2 cups, and requires special adaptors provided as accessories to the Genome Sequencer FLX Instrument.)

The procedure below indicates reagent quantities for *one emulsion reaction* (35×10^6 beads per LVE cup and 2.4×10^6 beads per SVE tube). However, due to the critical mechanical and physicochemical aspects of the emulsification process, you should process multiple reactions **in parallel**. Therefore, use the procedure below to form all the emulsions required for the sequencing experiment (up to 16 emulsions, as determined in section 2.3) **at the same time**.

1. Install the containers of Emulsion Oil securely in the TissueLyser.
   a. For LVE, use the special GS FLX Titanium emPCR Shaker Adapters, LV, provided as accessories to your Genome Sequencer FLX Instrument (use a counter balance if only a single emulsion cup is prepared).
   b. For SVE, place up to 8 tubes in the outer row of each of the two TissueLyser tube racks.

   Securing the cups or tube racks: The cups or tube racks must be firmly secured in the TissueLyser, as they will be shaken quite vigorously to mix the oil and create the emulsion. See the TissueLyser Operator's manual. For LVE, orient the cup lids toward the center of the TissueLyser.
2. Pre-mix the Emulsion Oil at **28 Hz for 2 minutes** for LVE (**25 Hz for 2 minutes** for SVE).

   Rattling sound: If the TissueLyser makes a loud rattling sound, turn it off immediately and make sure that both cups or tube racks are screwed tightly in place.
3. Remove the shaken Emulsion Oil (cups or racks of tubes) from the TissueLyser.
4. Add **5 ml** of 1× Mock Amplification Mix (from Section 3.1) to each cup of Emulsion Oil (or **290 µl** of 1× Mock Amplification Mix per tube of Emulsion Oil for SVE). Invert the cups (or the tube racks) 2-3 times to mix.
5. Place the cups or TissueLyser tube racks (in the proper orientation) back into the TissueLyser, and secure them firmly.
6. Set the TissueLyser to **28 Hz for 5 minutes** for LVE (**25 Hz for 5 minutes** for SVE), then press the start button to begin making the pre-emulsions.
7. When the TissueLyser stops shaking, remove the emulsions cups or TissueLyser tube racks as above.

▶

8 Add the captured library preparations from section 3.2.

a. For LVE, vortex and pour the whole content (about **3.75 ml**) from a 15 ml tube of captured library (from section 3.2) into each cup of pre-emulsion.

b. For SVE, pipette up and down and add the whole content (about **255 µl**) from a 1.7 ml tube of captured library (from section 3.2) to each tube of pre-emulsion.

c. Invert the cups (or the tube racks) 2-3 times to mix.

9 Place the cups or TissueLyser tube racks (in the proper orientation) back into the TissueLyser, and secure them firmly.

10 Set the TissueLyser to **12 Hz for 5 minutes** for LVE (**15 Hz for 5 minutes** for SVE), then press the start button to emulsify the emPCR amplification mixes.

- Again, if you hear a loud rattling noise, immediately stop the TissueLyser and secure the tube racks.
- This step will create an emulsion with aqueous phase microreactors that are the right size to contain single beads and amplification mix.

3.4 Amplification

After emulsification, each emulsion reaction (cup or tube) is split into individual amplification reactions, which will be pooled back together later for emulsion breaking and bead recovery (see section 3.5). The DNA amplification phase of the emPCR amplification process takes about 5 hours. After the amplification is complete, the emulsions may be left on the thermocycler for up to 16 hours at 10°C before you start isolating the beads, without affecting reaction efficiency.

Emulsion in tube caps: Be careful when opening the emulsion cups or tubes, as there may be emulsified material stuck to the underside of the caps; this material will be collected, but do NOT spin the emulsion tubes in an attempt to reclaim this material at this time as this would risk breaking the emulsions.

3.4.1 Dispensing the Emulsions (Controlled Room)

1. When the emulsification process is complete, remove the cups or TissueLyser tube racks from the TissueLyser, and open the emulsion containers carefully. See Caution, above.
2. Dispense the emulsified emPCR amplification mixes into 96-well thermocycler plates, *e.g.* using an Eppendorf Repeater Plus pipetter (100 µl per well).
   - For LVE, at 100 µl per well, each cup of emulsion will fill approximately 180 wells. A full LV emPCR kit worth of amplification reactions requires 4 plates.
   - For SVE, at 100 µl per well, each tube of emulsion will fill approximately 10 wells. A full SV emPCR kit worth of amplification reactions will usually fit in two plates. (Strip tubes may also be used if only a few emulsions are being prepared.)

   Sample cross-contamination risk: If you are processing distinct samples in separate cups or tubes, be careful not to contaminate them with one another; in particular, make sure to use a separate tip for each sample. Note however that multiple emulsion tubes of the same sample, *e.g.* if you are preparing samples for loading into a Medium (4 tubes) or a M/S (2 tubes) region gasket, are best transferred with a single tip.

   Some emulsion will have lodged on the underside of the cup lids or tube caps. Make sure to collect this material as well.
3. Check for the presence of air bubbles at the bottom of each well, as bubbles can cause breakage of the emulsion. If there are air bubbles, tilt the plate to dislodge them.

   Emulsion spillage: Carefully wipe off any emulsion that may have spilled on the surface of the plate during dispensing, especially if using PCR film to cap the wells. Failure to do this may result in improper sealing of the PCR reactions, or in broken emulsions.
4. Cap the wells and make sure that all the wells are properly sealed.
5. Clean up the area of any spilled reaction mix and turn on the UV light in the PCR enclosures before leaving the Controlled Room.

   Emulsion in caps: Always keep the PCR plates upright to prevent the wells' contents from lodging in the cap. If emulsions are lodged in the cap, the heated lid in the thermocycler can damage them.

3.4.2 Amplification Reaction (Amplicon Room)

1. Bring the plates containing the emulsified amplification reactions to the Amplicon Room, and place them in a thermocycler (may require multiple 0.2 ml blocks).
2. In the Setup file for the thermocycler, check to ensure that the lid is set to track within 5°C of the block temperature.
3. Set up and launch the following amplification program:
   a. 1× (4 minutes at 94°C)
   b. 50× (30 seconds at 94°C, 4.5 minutes at 58°C, 30 seconds at 68°C)
   c. 10°C until halted by user
4. 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. However, **DO NOT FREEZE** the DNA beads at any point in the process!
   - **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.

3.5 Bead Recovery

The difference in size of the LVE and SVE preparations is such that separate procedures were designed, which are optimized for the recovery of amplified beads from each size of emulsions.

- For LVE, a vacuum-assisted emulsion breaking and bead recovery set up allows convenient handling of the large number of DNA beads processed in the emulsion cups. This requires the **GS FLX Titanium emPCR Breaking Kits, LV, 12 pcs**; one set up is used per library sample, and can process any number of emulsion cups. This format is appropriate for samples being prepared to be loaded on Large region bead loading gaskets. This procedure is described in section 3.5.1.
- For SVE, the smaller number of beads processed in the emulsion tubes are best collected with a syringe-mounted filtration system. This requires the **GS FLX Titanium emPCR Filters, SV, 64 pcs kit**; one syringe + filter can process up to 4 emulsion tubes, so the process is appropriate for samples being prepared to be loaded on Medium, M/S, or Small region bead loading gaskets. This procedure is described in section 3.5.2; if you are processing SVE samples, skip section 3.5.1 entirely.

Broken emulsions: The emulsion should still be intact after the Amplification step. That is, the entire emulsion should be a uniform milky white suspension during and after amplification. If you notice that the lower phase is clear or see a clear droplet at the bottom of any of the amplification wells, the emulsion may have partially broken and resolved into its aqueous and oil phases. If you find this condition in a few wells, do not pool these suspect reactions with emulsions from other, unbroken reactions, as it is likely that many of the beads they contain will have a mixture of templates (*i.e.* are not clonally amplified). If the condition is more widespread, the amplified library may not be sequenceable.

For convenience (*e.g.* access to a centrifuge that can handle 50 ml conical tubes, for LVE bead recovery), the rest of the procedure may be carried out in a General Laboratory, rather than in the Amplicon Room.

Hazardous Chemical – Isopropanol Isopropanol is irritating to the eyes and skin and may cause drowsiness or dizziness if inhaled. It is highly flammable. Read the MSDS for handling precautions! Always wear eye protection, gloves, and a lab coat when handling this chemical. Always handle isopropanol in an appropriate fume hood.

3.5.1 Bead Recovery for Large Volume Emulsions (LVE)

3.5.1.1 Vacuum-Assisted Emulsion Breaking Set Up

1. Prepare 1× Enhancing Fluid TW and 1× Annealing Buffer TW from their concentrates, as described in sections 5.2.2 and 5.2.4:
   a. Add **187.5 ml** of Molecular Biology Grade Water to the **62.5 ml** of 4× 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 × Annealing Buffer TW stock (in its 250 ml container). Shake vigorously and keep on ice. This will be used later, in sections 3.6.1, 3.6.4, and 3.7.
2. Obtain a GS FLX Titanium emPCR Breaking Kit, LV, 12 pcs. See Figure 3–1 for a general view of an assembled set up.
   ▶ You will need one set up per library sample, for any number of cups processed for each given sample. However, you will need two 50 ml tubes (see next step, and section 3.5.1.2, step 5) for each emulsion cup worth of amplification reactions.
   Sample cross-contamination: If you prepared separate emulsion cups for more than one library sample, you MUST use a separate emulsion breaking set up for each one. Using a single emulsion breaking set up for multiple library samples would result in beads from the early samples contaminating the later ones.
3. Affix a standard 50 ml conical tube to each of the two lids, and set them in a tube rack so they are maintained upright. Keep the caps of the 50 ml tubes; you will need them later.
4. Insert the blue connector located at one end of the tubing into the top opening of the 8-pronged transpette.
5. Connect the other end of the tubing to a vacuum source. Make sure to include a liquid trap (*e.g.* an Erlenmeyer flask) in your vacuum system.
   Capturing isopropanol vapors: A liquid trap is required even if your vacuum source can handle carry-over vapors and liquids because a fair amount of isopropanol will be aspirated through the system, which will require capture for proper disposal.
   If a vacuum source is not available, it is also possible to aspirate the emulsions from the microtiter plate wells with a syringe and blunt needle , as described for the SVE procedure (section 3.5.2.1), and deliver the collected materials into the 50 ml tubes.

■

Figure 3–1: Schematic view of an assembled set up for vacuum-assisted emulsion breaking and bead recovery for Large Volume Emulsions

3.5.1.2 Emulsion Collection and Initial Washes for Large Volume Emulsions (LVE)

1. Turn on the vacuum source.

2. Aspirate the emulsions from 8 wells at a time, using a slow circular motion of the transpette tips at the bottom of the wells.
   - ▶ After aspirating all the emulsions, turn the transpette upside-down to help drain as much material as possible into the collection tubes.
   - ▶ The amplified DNA beads will collect in the two 50 ml tubes.

3. Rinse the plates **twice** with **100 µl** of isopropanol per well.
   - ▶ After each set, turn the transpette upside-down to help drain as much material as possible into the collection tubes.
   - ▶ Automation or mechanization of the isopropanol addition may be useful, to save time and labor.

   Amount of isopropanol: Do not overfill the wells with isopropanol. If you dispense a total of more than 40 ml of isopropanol during the two rinses of this step, the two 50 ml collection tubes will overflow and beads will be lost. Forty ml represents about two washes of two full plates, at 100 µl per well.

4. SLOWLY aspirate an additional (approximate) **5 ml** of isopropanol to collect any beads that may remain in the tubing.
   - ▶ Turn the transpette upside-down to help drain as much material as possible into the collection tubes, thus collecting as many beads as possible.

   Speed of aspiration: Do not aspirate too quickly at this step, as this could lead to splashing in the collection tubes and loss of beads into the vacuum system (trap).

5. If you are processing more than one emulsion cup of the SAME LIBRARY SAMPLE:
   a. Turn off the vacuum.
   b. Remove and cap the two 50 ml tubes containing the amplified DNA beads from the first emulsion cup (use the caps you set aside in section 3.5.1.1, step 3).
   c. Affix two new 50 ml tubes to the lids of the breaking set up, and set them in a tube rack so they are maintained upright. Again, keep the caps.
   d. Repeat steps 1 through 4 of this section for the sets of amplification reactions (usually two microtiter plates) of each emulsion cup.

   Sample cross-contamination: As remarked above, do not use a single emulsion breaking set up for multiple library samples. Doing so would result in beads from the early samples contaminating the later ones.

6. Turn off the vacuum.

7. Remove and cap the two 50 ml tubes containing the amplified DNA beads (use the caps you set aside in section 3.5.1.1, step 3, or in step 5, above). Discard the emulsion breaking set up.

■

3.5.1.3 Bead Washes and Recovery for Large Volume Emulsions (LVE)

1. 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.
2. Add isopropanol to final volume of **40 ml** in each tube. Vortex.
3. Pellet the beads in a centrifuge at **930 × *g* for 5 min** (2000 rpm in a Beckman Allegra centrifuge, rotor SX4750).
4. 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 **twice** with **35 ml** of isopropanol, by centrifugation as above.
   - ▶ Make sure to resuspend the bead pellets completely each time, to ensure proper rinsing of the beads. If vortexing is not sufficient, use a glass rod or a spatula to break bead aggregates.
5. Rinse the bead pellets once more, with **35 ml** of 1× Enhancing Fluid TW. The bead pellet will be softer after this wash, so pour the supernatant SLOWLY. Also, leave about 2 ml of 1× Enhancing Fluid TW, *i.e.* stop pouring when the white bead pellet starts coming off from the bottom of the tube.
6. 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 1× Enhancing Fluid TW left in the 50 ml tubes, you may have to centrifuge the 1.7 ml tubes (see step 8, below) and remove the supernatant one or more times to collect all the beads in the correct number of 1.7 ml tubes.
7. Rinse each of the 50 ml collection tubes with **600 µl** of 1× 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.
8. 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.
9. Remove the supernatant and rinse **twice** with **1 ml** of 1× Enhancing Fluid TW.
   - ▶ Make sure to resuspend the bead pellets completely each time, to ensure proper rinsing of the beads.
10. Resuspend the bead pellets in a final **1 ml** of 1× Enhancing Fluid TW.

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

■

3.5.2 Bead recovery for Small Volume Emulsions (SVE)

For SVE, use one syringe and filter for each sample, up to 4 emulsion tubes (40 amplification wells, at 100 µl per well) per syringe. This format conveniently handles samples being prepared for loading on Medium, M/S, or Small regions of a PicoTiterPlate device. The procedure is written for a single sample (syringe), but you can process as many in parallel as necessary; typical combination, for full PicoTiterPlate devices with these gaskets are: 4 syringes of 4 emulsions each, 8 syringes of 2 emulsions each, and 16 syringes of single emulsions.

3.5.2.1 Emulsion Collection and Initial Wash for Small Volume Emulsions (SVE)

1. Prepare 1× Enhancing Fluid TW and 1× Annealing Buffer TW from their concentrates, as described in sections 5.2.2 and 5.2.4:
   a. Add 187.5 ml of Molecular Biology Grade Water to the 62.5 ml of 4× 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× Annealing Buffer TW stock (in its 250 ml container). Shake vigorously and keep on ice. This will be used later, in sections 3.6.1, 3.6.4, and 3.7.
2. Screw a 16 gauge blunt, flat tip needle **directly** onto the end of a 10 ml syringe.
3. Draw the emulsion from each of the amplification wells from the sample you are processing into the syringe, up to the equivalent of 4 emulsion tubes.
   ► At 100 µl per amplification well, this would be about 40 wells.
   When combining multiple emulsions in one syringe, you may accidentally draw too much air. In that case, invert the syringe, draw some air to ensure all material is removed from the needle, and expel the excess air from the syringe (with the tip pointing up!). Then, proceed to draw in the remaining emulsions.
4. Add 100 µl of isopropanol to each well. Mix.
5. Draw the isopropanol rinse into the same syringe.
6. Draw a second time to collect the material that will have drained from the walls of the wells.
7. Invert the syringe and draw in 3 ml of air.
8. Remove and discard (sharps) the blunt needle.
9. Attach a filter from a GS FLX Titanium emPCR Filters, SV, 64 pcs kit to the syringe.
   Make sure to use blue filters from a GS FLX Titanium emPCR Filters, SV, 64 pcs kit. The GS FLX Titanium chemistry of the Genome Sequencer System does not use Swin-Lok filter holders (used with the Genome Sequencer FLX standard chemistry).
   Risk of cracking filter: Overtightening can crack the filter, rendering it inoperable. This can be detected by a clicking noise when the filter is affixed to the syringe. If you hear such a noise, replace the filter.
10. Place a Kimwipe on top of the vortex platform. Vortex the syringe for 5 sec at maximum speed, with the filter pointing down, onto the Kimwipe.
11. Gently expel the contents of the syringe through the filter, into a waste jar containing bleach.
    ► The library beads will be retained by the filter, while the emulsion oil is washed away with the isopropanol.
    If the syringe clogs, or you notice significant back pressure, draw air into the syringe until bubbles appear on top of the filter. Expel the contents of the syringe into the bleach jar. Repeat until all liquid is expelled from the syringe.
    Pressure buildup: If there is a buildup of pressure in the syringe during the wash, do NOT continue to expel the liquid, as this can cause breakage/leakage of the filter unit and consequent bead loss. Rather, draw air as noted above to dislodge the blockage and slowly expel the remaining liquid.

■

3.5.2.2 Bead Washes and Recovery for Small Volume Emulsions (SVE)

1. Wash the beads **three times** with isopropanol, as follows:
   a. Invert the syringe and draw in **3 ml** of air.
      ▶ This air space will allow for more efficient mixing.
   b. Draw in **3–5 ml** of fresh isopropanol.
      ▶ Do not draw in additional air after the isopropanol; this would displace the fluid from the surface of the membrane and interfere with proper mixing.
      ▶ Special case: if you are pooling 4 SVE reactions, the amplified emulsions will nearly fill the syringe (about 8 ml) so don't draw this additional isopropanol for the first wash.
   c. Place a Kimwipe on top of the vortex platform. Vortex the syringe for 5 sec at maximum speed, with the filter pointing down, onto the Kimwipe.
   d. Gently expel the contents of the syringe through the filter, into a waste jar containing bleach.

2. Wash the beads **three times** with 1× Enhancing Fluid TW, as follows:
   a. Invert the syringe and draw in **3 ml** of air.
      ▶ This air space will allow for more efficient mixing.
   b. Draw in **3 ml** of 1× Enhancing Fluid TW.
      ▶ Do not draw in additional air after the 1× Enhancing Fluid TW; this would displace the fluid from the surface of the membrane and interfere with proper mixing.
   c. Place a Kimwipe on top of the vortex platform. Vortex the syringe for 5 sec at maximum speed, with the filter pointing down, onto the Kimwipe.
   d. Gently expel the contents of the syringe through the filter, into a waste jar containing bleach.

3. Collect the washed beads from the filter by carrying out the following steps **twice**:
   a. Invert the syringe and draw in **3 ml** of air.
      ▶ This air space will allow for more efficient mixing.
   b. Draw in 1× Enhancing Fluid TW, to the **0.5 ml mark** of the syringe.
      ▶ Do not draw in additional air after the 1× Enhancing Fluid TW; this would displace the fluid from the surface of the membrane and interfere with proper mixing.
      ▶ Counting the volume inside the filter unit, this corresponds to ~**800 µl** of fluid.
   c. Place a Kimwipe on top of the vortex platform. Vortex the syringe for 5 sec at maximum speed, with the filter pointing down, onto the Kimwipe.
   d. Promptly invert the syringe and draw in another **3 ml** of air.
      ▶ This pulls the resuspended beads away from the filter.
   e. Remove the filter from the syringe (keep after the first collection; discard after the second collection).
   f. Dispense the bead suspension into a fresh 1.7 ml tube (both collections into the same tube).
   g. After the first collection, re-attach the filter onto the syringe; after the second collection, discard the syringe and the filter.

   If you drew more 1× Enhancing Fluid TW than indicated, the entire contents of the two collections may not fit into the tube; if that happens, pellet the beads in a minifuge (as described below), remove the supernatant, and dispense any remaining bead suspension from the syringe into the tube. It is important to recover as many beads as possible!

■

3.6 DNA Library Bead Enrichment

Hazardous Chemical – Sodium Hydroxide Solution Sodium hydroxide (present in the Melt Solution) is a highly corrosive chemical that may cause burns if it contacts eyes or skin. Reaction with water is exothermic. Read the MSDS for handling precautions! Always wear a lab coat, gloves and eye/face protection when handling sodium hydroxide solutions.

The LVE and SVE procedures merge back at this point. In both cases, the beads carry double-stranded clonal amplification products, and are in suspension in 1× Enhancing Fluid TW, in microcentrifuge tubes:

- For LVE, you should have two 1.7 ml tubes for each emulsion cup processed (typically, 4 tubes for a full kit).
- For SVE, you should have one 1.7 ml tube for each sample processed (up to 4 SVE emulsion tubes per sample; 4 to 16 tubes of beads for a full SV emPCR kit).
- The reagent volumes listed in this section apply to each tube of beads.

3.6.1 Preparation for Indirect Enrichment

1. Turn on the heating dry-block and set it to 65°C.
2. If necessary, prepare a stock of Melt Solution by mixing **125 µl** of NaOH (10 N) in **9.875 ml** of Molecular Biology Grade Water, as described in section 5.2.3. This solution will be used in this section and in section 3.6.4, below; it has a shelf life of only 7 days (at +2 to +8°C).
3. Pellet the collected DNA beads from either the LVE or SVE Bead Recovery step (section 3.5) in a bench top minifuge, as follows:
   I. spin for 10 seconds,
   II. rotate the tube 180°, and
   III. spin again for 10 seconds.
4. Remove and discard the supernatants.
5. 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 minutes.
   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.
6. Wash the bead pellets **twice** by centrifugation as above, with **1 ml** of 1× Annealing Buffer TW per tube each time. The 1× Annealing Buffer was prepared in section 3.5.1.1, step 1.b.

7 (Optional) It may be useful to determine the % bead recovery at this intermediate point, to make sure that you did not lose too many beads along the way, or for troubleshooting purposes in case you obtain too few enriched beads at the end of the procedure. To determine the % bead recovery, do the following:

a. Resuspend the bead pellet by adding **1 ml** (**200 µl** for SVE) of 1× Annealing Buffer TW Vortex.

b. Draw a 3 µl aliquot of the bead suspension and count the beads using the settings for your particle counter given in section 5.3 (note new shape factor of 1.1 with the Coulter Counter Multisizer 3). A recovery of over 85% (65% for SVE) would be typical at this point of the procedure. Calculate the % bead recovery using following equation:

$$\%\ \text{Bead Recovery} = \frac{\text{Number of beads recovered}}{\text{total input beads*}} \times 100$$

* 35×10^6 beads for one emulsion cup, or 2.4×10^6 beads per emulsion tube included in this sample

Since the particle counter provides results as a concentration (beads / volume), the determination of the "Number of beads recovered" for the equation above requires an estimation of the total volume of the bead suspension. Using simply the volume of 1× Annealing Buffer TW added to the bead pellet (1 ml or 200 µl) would result in an underestimation of this volume because the bead pellet itself is substantial.

1. A simple way to assess the volume of suspension is to draw the suspension into a pipettor set to a volume smaller than the actual volume of suspension, then dial up the pipettor until all the suspension has been drawn in and take a reading. (For LV, this requires a 2 ml pipettor.)
2. To avoid losing beads into the pipette tip, an alternative method is to weigh the tube containing the bead suspension on a balance tared with an empty tube of the same type, and calculate the volume assuming that the suspension has a density of 1.0 g/ml.

c. Pellet the beads again in a bench top minifuge, as follows:
   I. spin for 10 seconds,
   II. rotate the tube 180°, and
   III. spin again for 10 seconds.
   IV. Remove and discard the supernatant

If performing enrichment titrations and the percent bead recovery is less than 80%, do not proceed and repeat the emulsion set-up.

8 Add **45 µl** per tube (**30 µl** for SVE) of 1× Annealing Buffer TW, and **25 µl** per tube (**6 µl** per emulsion tube equivalent, in each tube of SVE beads) of Enrichment Primer. Vortex to mix completely.

9 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**.

10 Add **800 µl** of 1× Enhancing Fluid TW. Vortex.

11 Pellet the beads by centrifugation as above, and remove the supernatants.

12 Wash the bead pellets **twice** more by centrifugation, with **1 ml** of 1× Enhancing Fluid TW per tube each time.

13 After removing the supernatants, resuspend each bead pellet in **800 µl** of 1× Enhancing Fluid TW. Vortex.

3.6.2 Preparation of the Enrichment Beads

1. Vortex the tube of Enrichment Beads for **1 minute** to resuspend its contents completely.
   - If you are not processing a full emPCR Kit, you may aliquot out the amount of Enrichment Beads suspension needed for your preparation, and re-freeze the rest. You will need:
     - **160 µl** per LVE emulsion cup processed;
     - **20 µl** per SVE emulsion tube processed.
   - You can prepare all the Enrichment Beads in bulk; they will be aliquoted when used (section 3.6.3).
2. Place the tube of Enrichment Beads in a Magnetic Particle Collector (MPC), pelleting the paramagnetic Enrichment Beads against the side of the tube.
3. Remove and discard the supernatant, taking care not to draw off any Enrichment Beads.
4. Wash the pelleted beads **twice** with **1 ml** of 1× 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 1× Enhancing Fluid TW (or the same volume that you aliquoted out; see step 1, above). Vortex.

■

3.6.3 Enrichment of the DNA-Carrying Beads

1. Add **80 µl** (**20 µl** per emulsion tube processed for SVE) of washed Enrichment Beads (from section 3.6.2) to each tube of amplified DNA beads (from section 3.6.1).
2. Vortex to mix completely the DNA Beads and the Enrichment Beads.
3. Rotate on a LabQuake tube roller at ambient temperature (+15°C to +25°C) 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.
   - Optionally, collect the supernatants containing “anti-enriched” beads (those that carry no amplified DNA and are removed by the enrichment process), for troubleshooting in case the sequencing reaction shows low numbers of keypass reads.
6. Wash the beads with **1 ml** of 1× 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** of 1× 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.

■

3.6.4 Collection of the Enriched DNA Beads

Hazardous Chemical – Sodium Hydroxide Solution Sodium hydroxide (present in the Melt Solution) is a highly corrosive chemical that may cause burns if it contacts eyes or skin. Reaction with water is exothermic. Read the MSDS for handling precautions! Always wear a lab coat, gloves and eye/face protection when handling sodium hydroxide solutions.

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 (pairs of tubes corresponding to one emulsion cup for LVE; individual tube corresponding to 1-4 emulsion tubes for SVE), to a separate 1.7 ml microfuge tube.
4. Pellet the enriched DNA beads by centrifugation as before (spin 10 seconds, rotate the tube 180°, and spin again 10 seconds). Remove and discard the supernatant. (This step is not necessary for SVE).
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).
6. Discard the tubes of spent Enrichment Beads.
7. Pellet the enriched DNA beads by centrifugation as before. Remove and discard the supernatants.
8. Wash the bead pellets **three times** with **1 ml** of 1× Annealing Buffer TW (by centrifugation) to completely neutralize the Melt Solution.
9. Resuspend each of the final bead pellets in **200 µl** (**30 µl** per SVE emulsion tube equivalent) of 1× Annealing Buffer TW.

3.7 Sequencing Primer Annealing

1. Add **50 µl** (**6 µl** per SVE emulsion tube 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**.
3. Add **800 µl** of 1× Annealing Buffer TW, pellet the beads as before, and remove the supernatant.
4. Wash each of the bead pellets **twice** with **1 ml** (**500 µl** for SVE) of 1× Annealing Buffer TW.
5. Resuspend each of the final bead pellets by adding **1 ml** (**100 µl** for SVE) of 19 Annealing Buffer TW.

6 To determine the % bead enrichment, do the following:

a. Count a 3 µl aliquot of the beads with a particle counter, following the manufacturer's instructions and using the settings listed in section 5.3.

b. Calculate the % bead enrichment using the following equation:

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

* 35×10^6 beads per emulsion cup and 2.4×10^6 beads per emulsion tube included in this sample

Since the particle counter provides results as a concentration (beads / volume), the determination of the "Number of beads recovered" for the equation above requires an estimation of the total volume of the bead suspension. Using simply the volume of 1× Annealing Buffer TW added to the bead pellet (1 ml or 200 µl) would result in an underestimation of this volume because the bead pellet itself is substantial.

1. A simple way to assess the volume of suspension is to draw the suspension into a pipettor set to a volume smaller than the actual volume of suspension, then dial up the pipettor until all the suspension has been drawn in and take a reading. (For LV, this requires a 2 ml pipettor.)
2. To avoid losing beads into the pipette tip, an alternative method is to weigh the tube containing the bead suspension on a balance tared with an empty tube of the same type, and calculate the volume assuming that the suspension has a density of 1.0 g/ml.

c. Percent enrichment values between 5 and 15% are indicative of libraries that usually yield good sequencing results.

Bubbles and bead counting: The presence of air bubbles in the diluted bead suspension may interfere with bead counting (bubbles may be counted as beads by the particle counter instrument). To minimize this risk, mix the beads (*e.g.* in the Isoton II solution) by swirling gently rather than shaking or vortexing.

7 Store the beads (immobilized, clonally amplified DNA library) at +2°C to +8°C. The stored beads will remain sequenceable for at least 1 month.

- The bead count will vary depending on the efficiency of the emPCR amplification reaction and the enrichment, but typical recoveries for enriched beads range from 5 to 15% of the original bead input. If you observe an unusually low or high recovery of enriched DNA beads following the emPCR amplification process, it is best to repeat the emPCR amplification with more or less input DNA (*e.g.* a two-fold factor) because:
  - Low recovery may generate too few beads to take full advantage of the Genome Sequencer System's ultra-high throughput. (Note, however, that these beads would typically generate very high quality reads, which may be more important than the number of reads for certain experiments.)
  - A high recovery, on the other hand, may indicate that more than one effective DNA molecule is present in a large proportion of the beads, which would result in a lot of rejected "mixed" reads.
- If the emPCR amplification procedure did not produce the number of beads suggested for loading into the region size you intend to use for your sequencing Run (see the *GS FLX Titanium Sequecing Method Manual*), the beads obtained may still be worth sequencing. In particular, a yield as low as 5 or 6% enriched beads would correspond to fewer beads than recommended for the medium and M/S region sizes, but would still normally provide acceptable sequencing results; an emPCR amplification yield of 10% enriched beads produces plenty of beads for all region sizes.

4. Where to Go from Here

When you have completed the above procedures, the sample is ready for the sequencing phase of the Genome Sequencer System. In practice, this means that the DNA sample, previously prepared into a library of DNA fragments using the appropriate GS FLX Titanium series procedure has now been:

- immobilized onto microspheres (beads),
- clonally amplified, and
- annealed with the sequencing primer.

The result is a sequencing-ready library of single-stranded DNA beads, each carrying from 10 to 50 $\times$ 10^6 copies of a single DNA fragment from the original library. The amount of immobilized, amplified library prepared should be appropriate for the size of the experiment being performed (*e.g.* size of the genome to be sequenced and required depth of coverage; or the number and size of PicoTiterPlate regions to be loaded with this preparation).

In the next phase of the experiment, you will load your sample, along with other reagents, in the wells of a PicoTiterPlate device; and perform the sequencing with an appropriate GS FLX Titanium Sequencing and PicoTiterPlate Kits, and a Genome Sequencer FLX Instrument. This is described in detail in the *GS FLX Titanium Sequencing Method Manual*.

5. Appendix

5.1 Table of Materials Required but Not Provided

Some pieces of equipment or standard laboratory supplies, as well as some third party reagents, are not provided but are required to carry out the emPCR amplification procedures described in this manual. These items are listed in Table 5 –1. Make sure that all necessary items are available prior to beginning an experiment.

The specific items identified by Source and Ref. Number in Table 5 –1 are examples (available in North America) that have been demonstrated to work well in the procedure, though alternatives may also perform adequately. Please contact your Roche Representative for any questions regarding these items or possible substitutions.

Equipment	Quantity Required	Source	Ref. Number
Thermocycler	1–4[a]	Many possible	N/A
96-well, 0.2 ml block for Thermocycler	4[a]	Many possible	N/A
Minifuge: ▶ 2 in Controlled Room ▶ 1 in Amplicon Room	3	Many possible	N/A
Vortex Genie ▶ 2 in Controlled Room ▶ 1 in Amplicon Room	3	Many possible	N/A
TissueLyser II	1	Qiagen	85300
TissueLyser tube rack assemblies	2	Qiagen	69982
LabQuake Roller	1	Thermolyne	400110
PCR Enclosure (PCR preparation)	2	Fisher Scientific	16-108-137
Vented Hood (dedicated for emulsion breaking only)	1	Many possible	N/A
Particle Counter	1	Beckman Coulter	Multisizer 3 or Z2, or Z1
		Nexcelcom	Cellometer Auto T4
Heating Dry-block	1	Many possible	N/A
Centrifuge[c]	1	Beckman Coulter	X-12 or X-15 392472
Centrifuge rotor with swinging buckets[c]	1	Beckman Coulter	SX4750A 369704
50 ml Tube adaptors for swinging bucket rotor[c]	2	Beckman Coulter	359474
Vacuum pump (with moisture trap)[c]	1	GAST	ROA-P101-AA

(cont'd)

Lab Supplies	Quantity Required	Source	Ref. Number
Full set of micropipettes (2–1000 µl): ▶ 2 sets in Controlled Room ▶ 1 set in Amplicon Room	3 sets	Rainin	RL Series
Pipette Tips	1 box each	Rainin	RT-LxF series
Repeater plus pipette	1	Eppendorf	2226020-1
1.0 sterile combitip plus tips	1	Eppendorf	2226730-2
1.5 ml Low-adhesion microcentrifuge tubes	1 bag	DOT Scientific	RN 1700-GNB
2.0 ml tubes, Snaplock attached cap, boil-proof, Clear[b]	1 bag	DOT Scientific	608-GMT
Balance (0.01 g precision; optional)	1	Many possible	N/A
Glass rod (optional)	1	Many possible	N/A
Spatula (optional)	1	Many possible	N/A
96-well plates, semi skirted	2 plates	Fisher Scientific	E951020303
Adhesive PCR Film[c]	2	ABgene	AB-0558
Cap strips for 96-well plates	2	Fisher Scientific	E0030127498
15 ml Conical Falcon Tube	2	Falcon, Becton Dickenson	2196
50 ml Conical tube[c]	4	Fisher Scientific	14-432-22
Magnetic Particle Concentrator	1	Invitrogen	123-21D
10 ml Disposable Syringe with Luer-Lok[b]	1 – 16	Fisher Scientific	14-823-2A
		B. Braun	4606728 V
Blunt, flat tip needle, 16 gauge[b]	1 – 16	Brico Medical Supplies	BN1615
		Tyco Healthcare	8881-202322
		VWR	MJ8881-202322
Beckman Coulter CC Size Standard L10, 15 ml (if calibration is needed)[d]	2 drops	Beckman Coulter	6602796
Isoton II[d]	20 ml	Beckman Coulter	8546719
Accuvettes[d]	1	Beckman Coulter	8320592
Cellometer disposable chamber-slide[e]	2 slides	Nexcelom	CHT4-003

(cont'd)

Reagents/Consumables	Stock Conc.	Quantity Required [e]	Source	Ref. Number
Molecular Biology Grade Water	NA	300 ml	Fisher Scientific (Eppendorf)	E0032006205
2-Propanol (Isopropanol)	100%	25 ml/rxn	Fisher Scientific	A426P-4
NaOH	10 N	125 µl	Fisher Scientific	SS255-1

Table 5–1: Additional materials required for the emPCR amplification procedure (not supplied)

[a] Four 96-well thermocycler blocks are required to process a full kit; these can be used on single-block or on multi-block thermocycler models.

[b] These items are used only in the Small Volume Emulsion emPCR amplification procedure.

[c] These items are used only in the Large Volume Emulsion emPCR amplification procedure.

[d] These items are used only with the particle counters from Beckman Coulter.

[e] This item is used only with the particle counter from Nexcelom.

Items identified under "Source" (and corresponding Ref. Numbers) are examples only.

For a complete overview of related products and manuals, please visit and bookmark our home page at **www.roche-applied-science.com**, and our Special Interest Site on the Genome Sequencer System at **www.genome-sequencing.com**.

5.2 User-Prepared Reagents

5.2.1 1× Capture Bead Wash Buffer TW

Ingredient	10 ml Preparation
10× Capture Bead Wash Buffer TW	1.0 ml
Molecular Biology Grade Water	9.0 ml

1. Place 1.0 ml of 10× Capture Bead Wash Buffer TW in a 15 ml conical centrifuge tube, and add 9.0 ml of Molecular Biology Grade Water. Cap the tube, and invert it several times to mix.
2. Label the tube "1× Capture Bead Wash Buffer TW". Store at +2°C to +8°C.

Shelf life: 1× Capture Bead Wash Buffer TW has a shelf life of 90 days at +2°C to +8°C; indicate the expiration date on the container, and do not use after the expiration date has passed.

5.2.2 1× Enhancing Fluid TW

Ingredient	250 ml Preparation
4× Enhancing Fluid TW	62.5 ml
Molecular Biology Grade Water	187.5 ml

1. Add 187.5 ml of Molecular Biology Grade Water to the 62.5 ml of 4× Enhancing Fluid TW stock (in its 250 ml container). Swirl to mix.
2. Mark on the container (label on the back) that this reagent has been diluted to its 1× working concentration. Store at +2°C to +8°C.

Shelf life: 1× Enhancing Fluid TW has a shelf life of 30 days at +2°C to +8°C; indicate the expiration date on the container, and do not use after the expiration date has passed.

5.2.3 Melt Solution

Hazardous Chemicals – Sodium Hydroxide Solution (10 N) Sodium hydroxide is a highly corrosive chemical that may cause burns if it contacts eyes or skin. Reaction with water is exothermic. Read the MSDS for handling precautions! Always wear a lab coat, gloves and eye/face protection when handling 10 N solution of sodium hydroxide.

Ingredient	10 ml Preparation
NaOH (10 N)	0.125 ml
Molecular Biology Grade Water	9.875 ml

1. In a 15 ml conical centrifuge tube, add 9.875 ml of water first, then 0.125 ml of 10 N NaOH. Cap the tube, and invert it several times to mix.
2. Store at +2°C to +8°C.

Shelf life: Melt Solution has a shelf life of 7 days at +2°C to +8°C; indicate the expiration date on the container, and do not use after the expiration date has passed.

5.2.4 1× Annealing Buffer TW

Ingredient	80 ml Preparation
10× Annealing Buffer TW	8 ml
Molecular Biology Grade Water	72 ml

1. Add 72 ml of Molecular Biology Grade Water to the 8 ml of 10× Annealing Buffer TW stock (in its 250 ml container). Swirl to mix.
2. Mark on the container (label on the back) that this reagent has been diluted to its 1× working concentration. Store at +2°C to +8°C.

Shelf life: 1× Annealing Buffer TW has a shelf life of 30 days at +2°C to +8°C; indicate the expiration date on the container, and do not use after the expiration date has passed.

5.3 Bead Counter Settings

454 Life Sciences Corporation, a Roche company, has validated the use of 4 devices for the quantitation of amplified DNA library beads obtained from emPCR amplification reactions: the Coulter Counter Multisizer 3, Z2, and Z1 models from Beckman Coulter, and the Cellometer Auto T4 from Nexcelom. Figure 5–1 shows all the settings for the Multisizer 3, entered through the instrument's user interface; the settings for the Coulter Counter Z2 and Z1 models are listed in Table 5–2; and those for the Cellometer Auto T4 are shown in Figure 5–2 (see your instrument's manufacturer documentation for operation details, in particular concerning the risk of capillary obstruction, for the Coulter Counter instruments).

Bubbles and bead counting: The presence of air bubbles in the diluted bead suspension may interfere with bead counting when using any of the Coulter Counter instruments (bubbles may be counted as beads). To minimize this risk, mix the beads in the Isoton II solution by swirling gently rather than shaking or vortexing.

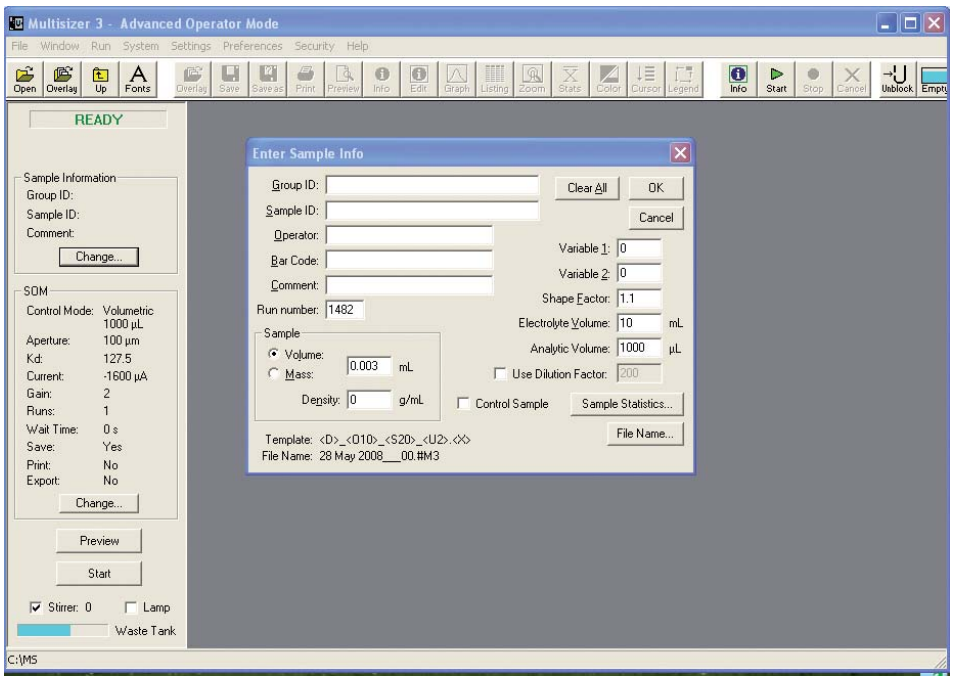


Figure 5–1: Coulter Counter Multisizer 3 settings for bead counting

The Figure shows appropriate settings for the quantitation of amplified DNA library beads obtained from emPCR amplification reactions: see the "SOM" box to the left; and the "Enter Sample Info" window, which is accessed by clicking the Change button.

Parameter	Settings	
	Dual Thresholds	**Single Threshold**
Upper Size (T_u)	25 µm	--
Lower Size (T_l)	15 µm	15 µm
Count Mode	Between	Above T_l
Aperture	100 µm	100 µm
Metered Volume	1.0	1.0
Result Type	Concentration	Concentration
Dilution Factor	3333	3333

Table 5–2: Coulter Counter Z2 or Z1 models settings for bead counting

The Table shows appropriate settings for the quantitation of amplified DNA library beads obtained from emPCR amplification reactions. Dual thresholds is preferable, if available.

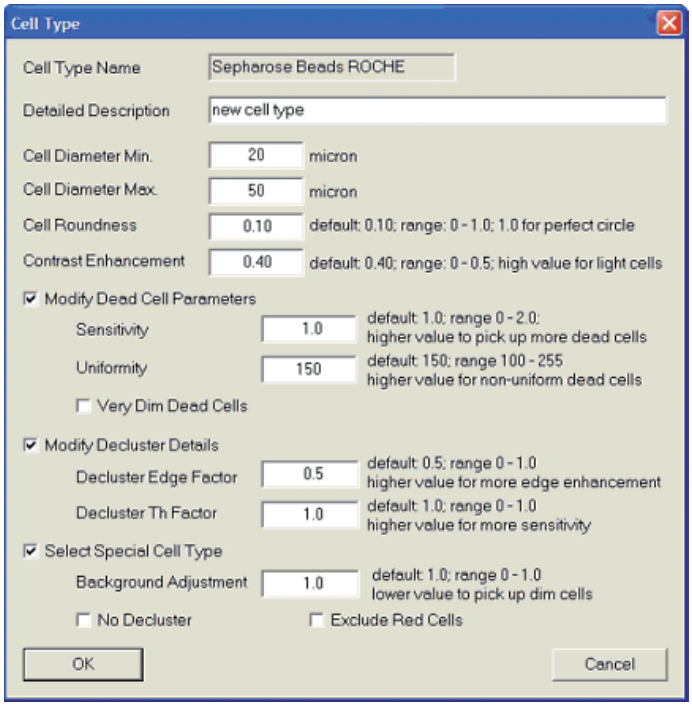


Figure 5–3: Cellometer Auto T4 settings for bead counting

The Figure shows appropriate settings for the quantitation of amplified DNA library beads obtained from emPCR amplification reactions.

5.4 Additional Information

5.4.1 Revision History

Manual Version	Instrument Version	Software Version	Revision Date
FLX.01 – USM-00014.K	GS FLX	1.1.01	December 2006
FLX.02 – USM-00014.L	GS FLX	1.1.02	June 2007
FLX.03 – USM-00033.A	GS FLX	1.1.03	December 2007
FLX.Ti.00 – USM-00054.A	GS FLX	2.0.00	October 2008

Every effort has been made to ensure that the information contained in this document was correct at the time of printing. However, 454 Life Sciences Corporation and Roche Diagnostics GmbH reserve the right to make corrections, clarifications, updates, or any other changes deemed necessary without notice, as part of ongoing product development.

 Questions or comments regarding the contents of this manual can be directed to the address below or to your Roche Representative.

454 Life Sciences Corporation
1 Commercial St.
Branford, CT
USA 06405

5.4.2 Related Publications

A full suite of publications are available that describe in detail the components and usage of the Genome Sequencer System:

- *Genome Sequencer FLX Operator's Manual (October 2008)*
- *Genome Sequencer FLX Titanium Applications and Methods Manual*, including:
  - *GS FLX Titanium General Library Preparation Method Manual*
  - *GS FLX Titanium emPCR Method Manual* – this manual
  - *GS FLX Titanium Sequencing Method Manual*
  - A Quick Guide version of each method is also included.
- *Genome Sequencer Data Analysis Software Manual*
- *Genome Sequencer System Site Preparation Guide (October 2008)*

Note also that some of the applications of the Genome Sequencer FLX standard System (December 2007) are not yet available for the GS FLX Titanium chemistry. These include the preparation and usage of Paired End and Amplicon libraries, and the usage of Multiplex Identifiers (MIDs). For these applications, the Genome Sequencer FLX standard System methods and kits must still be used. The December 2007 GS FLX manual set comprises the following:

- *Genome Sequencer FLX Operator's Manual (December 2007)*
- *Genome Sequencer FLX System Methods Manual*, including:
  - *GS FLX Shotgun DNA Library Preparation Method Manual*
  - *GS FLX Paired End DNA Library Preparation Method Manual*
  - *GS FLX Amplicon DNA Library Preparation Method Manual*
  - *GS FLX emPCR Method Manual* – this manual
  - *GS FLX Sequencing Method Manual*
  - A Quick Guide version of each Method is also included.
- *Genome Sequencer FLX Data Analysis Software Manual*
- *Genome Sequencer System Site Preparation Guide (December 2007)*

All Genome Sequencer Manuals, Guides and Bulletins are available three ways: in hardcopy form, on a CD from your Roche representative, or downloaded from the customer restricted access area of **www.genome-sequencing.com**.

5.4.3 Intended Use

The Genome Sequencer System is intended for life science research applications and must be used exclusively by laboratory professionals trained in laboratory techniques and having studied the instructions for the use of its various associated kits.

5.4.4 Notice to Purchaser

RESTRICTION ON USE: Purchaser is only authorized to use the Genome Sequencer Instrument with PicoTiterPlate devices supplied by 454 Life Sciences Corporation and in conformity with the operating procedures contained in the Genome Sequencer System manuals and guides.

Made in USA by 454 Life Sciences Corporation, Branford, CT, USA, a Roche company.

For life science research only. Not for use in diagnostic procedures.

5.4.5 Trademarks

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5.4.6 Assistance

If you have questions or experience problems with the Genome Sequencer System, please call, write, fax, or email us.

When calling for assistance, be prepared to provide the serial number of your Genome Sequencer Instrument and/or lot number of the kit(s) you are using. The instrument's serial number is located on the label found on the back of the instrument cart.

If you are located in…	**Please contact Roche Applied Science Technical Support via:**	
USA or Canada	phone: 1-800-262-4911 (toll-free)	e-mail: **us.gssupport@roche.com**
Europe, Middle East, Asia Pacific, Mexico, South America or Africa	phone: +49-8856-60-6457 or toll-free +800SEQUENCE	e-mail: **service.sequencing@roche.com**
Japan	phone: +03-5443-5287	e-mail: **tokyo.biochemicals@roche.com**

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