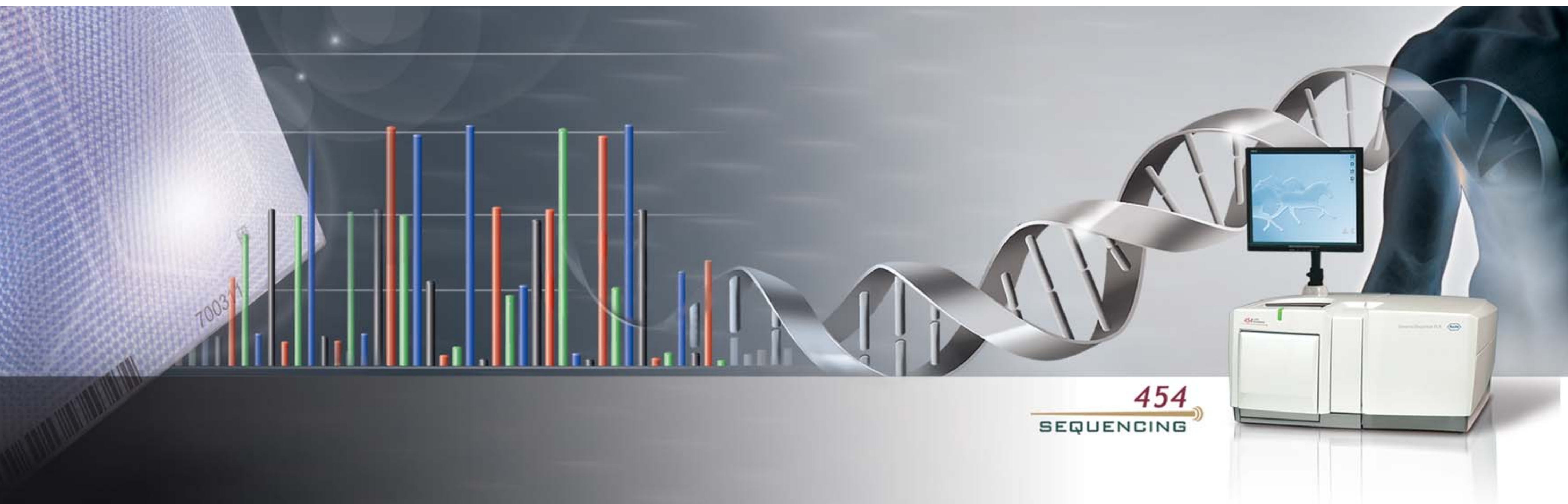
Roche

Amplicon Library Preparation Method Manual

GS FLX Titanium Series

October 2009



1. Workflow

The procedure to prepare Amplicon libraries is shown in Figure 1. It consists of a PCR amplification, performed using special Fusion Primers for the Genome Sequencer FLX System (see Sections 2 and 4.1) The method provides for the preparation of just a few or of a large number of Amplicons at a time, in individual PCR tubes or in 96-well plates.

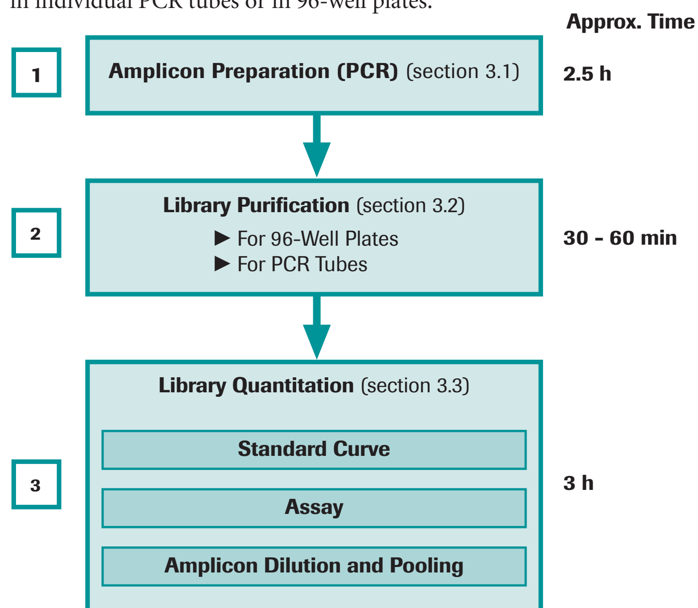


Figure 1: Workflow of the Amplicon library preparation method.

2. Before You Begin

Experimental set up for sequencing an Amplicon library can be complex. See the relevant sections of the *Genome Sequencer System Research Applications Guide* and the *Genome Sequencer System Introduction Manual*. All materials used to create the Amplicons that will constitute the library must be obtained from 3rd party vendors, including the forward (A) and reverse (B) fusion primers.

This procedure requires 5 – 20 ng (genomic DNA) or 1 – 2 ng (plasmid DNA or similar) of starting DNA material, in 1 µl of molecular biology grade water.

PCR optimization: Amplification of any given target sequence may require individual testing and optimization. See Appendix, Section 4.1.

3. Procedure

3.1 Amplicon Preparation (PCR)

1. Prepare the PCR Master Mix. Table 1 gives the volumes for 1, 8 or 96 Amplicons. Make the quantity appropriate for the number of Amplicons included in your experimental design.

Reagent	1 Amplicon		8 Amplicons		96 Amplicons	
Forward Primer (10 µM)	1	µl	8.8	µl	105.6	µl
Reverse Primer (10 µM)	1	µl	8.8	µl	105.6	µl
dNTP mix (10 mM each)	0.5	µl	4.4	µl	52.8	µl
FastStart 10 × Buffer #2	2.5	µl	22	µl	264	µl
FastStart HiFi Polymerase (5 U/µl)	0.25	µl	2.2	µl	26.4	µl
Molecular Biology Grade Water	18.75	µl	165	µl	1980	µl
Total	**24**	**µl**	**211.2**	**µl**	**2534.4**	**µl**

Table 1: Composition of the PCR Master Mix.
The columns for 8 and 96 Amplicons make 10% extra mix; the totals have been rounded.

2. Dilute the DNA sample(s) to the appropriate concentration, in molecular biology grade water.
   a. Genomic DNA: dilute to 5 – 20 ng/µl
   b. Plasmid DNA: dilute to 1 – 2 ng/µl
3. Dispense 24 µl of PCR Master Mix for the number of Amplicons you are preparing. Depending on the number of Amplicons you are preparing, you can do this in PCR tubes or in 96-well PCR plates. (See Appendix, Section 4.2, for recommended plate layout.)
4. To each 24 µl of PCR Master Mix, add 1 µl of a diluted DNA sample.
5. Seal the plate carefully and place it in a thermocycler.
6. Run the PCR program; the conditions below are guidelines only (see Note on PCR Optimization in the Before You Begin section).
   - 1 ×: 94°C, 3 min
   - 25 to 35 ×:
     - 94°C, 15 sec
     - 55 – 65°C, 45 sec
     - 72°C, 1 min
   - 1 ×: 72°C, 8 min
   - 4°C on hold

3.2 Library Purification

3.2.1 For 96-Well Plates

It is recommended to process one plate at a time. See Appendix, Section 4.2, for recommended plate layout.

1. Set a heat block to 40°C.
2. Prepare 25 ml of 70% ethanol, by adding 17.5 ml of 100% ethanol to 7.5 ml of Molecular Biology Grade Water, and vortex.
3. Centrifuge the plate with PCR products (your Amplicons) for **30 sec at 900 × *g***.
4. Pipet **22.5 µl** of molecular biology grade water into each well of a new 96-well, round bottom, polypro-pylene (PP) plate.
5. Carefully transfer **22.5 µl** of each PCR product from the PCR plate to each well of the PP plate.
6. Vortex the AMPure bead bottle for 20 seconds, or until the beads are completely resuspended.
7. Add **72.0 µl** of AMPure beads to each well and mix thoroughly by pipetting up and down **at least 12 times**, until the mixture is homogeneous.
8. Incubate for **10 min** at room temperature.
9. Place the plate on the 96-well magnetic ring stand and incubate for **5 min** at room temperature, until the supernatant is clear.
10. With the plate still on the magnetic ring stand, carefully remove and discard the supernatant without disturbing the beads.
11. Remove the plate from the magnetic ring stand and add **100 µl** of 70% ethanol (freshly prepared in step 2) to each well.
12. Tap the plate **10 times** to agitate the solution. The pellet may not resuspend completely; this is acceptable.
13. Place the plate on the magnetic ring stand and incubate for **1 min**.
14. With the plate still on the magnetic ring stand, carefully remove and discard the clear supernatant without disturbing the beads.
15. Repeat steps 11– 14. Remove as much of the supernatant as possible.
16. Place the plate and magnetic ring stand together on a heat block set at 40°C until all pellets are completely dry (**10 – 20 min**). Do not leave the plate on the heat block longer than necessary to avoid overdrying.
17. Carefully remove the plate from the heat block, keeping it on the magnetic ring stand to ensure that the pellets are stable during transfer.

18. Add **20 µl** of 1× TE to each well. Remove from the ring stand. Tap the plate gently until all pellets are resuspended.
19. Place the plate over the magnetic ring stand and move it in a circular motion to dislodge the pellet ring. Tap the plate again until all pellets are dispersed. This ensures efficient elution of the PCR products from the beads.
20. Place the plate on the magnetic ring stand and incubate for **2 min**.
21. Transfer the supernatant from each well into a fresh 96-well PCR plate. It is difficult to avoid any transfer of pellet from some of the wells; this is acceptable.
22. Seal the plate and store at -20ºC until ready to proceed to the quantitation step, Section 3.3.

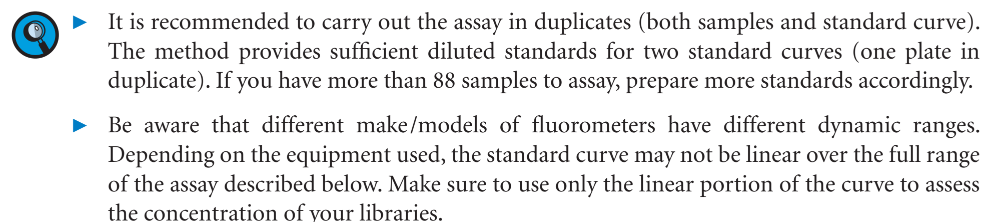
3.2.2 For PCR Tubes

1. Set a heat block to 37°C.
2. Prepare 70% ethanol in the amount needed (400 µl per Amplicon). For 10 ml, add 7 ml of 100% ethanol to 3 ml Molecular Biology Grade Water, and vortex.
3. Briefly centrifuge the PCR tubes.
4. Pipet **22.5 µl** of molecular biology grade water into 1.5 ml tubes (one tube per Amplicon).
5. Transfer **22.5 µl** of each PCR product from the PCR tubes to each 1.5 ml tube.
6. Vortex the AMPure bead bottle for 20 seconds, or until the beads are completely resuspended.
7. Add **72.0 µl** of AMPure beads to each tube, and mix thoroughly by vortexing for **5 sec**.
8. Incubate for **10 min** at room temperature.
9. Place the tubes in an Magnetic Particle Collector (MPC) and incubate for **5 min** at room temperature.
10. With the tubes still in the MPC, carefully remove and discard the supernatant without disturbing the beads.
11. Remove the tubes from the MPC and add **200 µl** of 70% ethanol (freshly prepared in step 2) to each tube.
12. Vortex the tubes for **5 sec**. The pellet may not resuspend completely; this is acceptable.
13. Place the tubes on the MPC and incubate **1 min**.
14. With the tubes still on the MPC, carefully remove and discard the supernatant without disturbing the beads.

15. Repeat steps 11 – 14. Remove as much of the supernatant as possible.
16. Place the open tubes on a heat block set at 37°C until the pellet is completely dry (about **5 min**). Do not leave the tubes on the heat block longer than necessary to avoid overdrying.
17. Remove the tubes from the MPC.
18. Add **10 µl** of 1× TE to each tube. Vortex **5 sec** or until the pellet is completely resuspended.
19. Place the tubes in the MPC and incubate for **2 min** at room temperature.
20. With the tubes still in the MPC, carefully transfer the supernatants to a set of fresh screw cap o-ring 1.5 ml tubes.
21. Store the purified Amplicons individually at -20°C until ready to proceed to the quantitation step, Section 3.3.

3.3 Library Quantitation

Library quantitation is done by fluorometry using the Quant-iT PicoGreen dsDNA Assay Kit.



- It is recommended to carry out the assay in duplicates (both samples and standard curve). The method provides sufficient diluted standards for two standard curves (one plate in duplicate). If you have more than 88 samples to assay, prepare more standards accordingly.
- Be aware that different make/models of fluorometers have different dynamic ranges. Depending on the equipment used, the standard curve may not be linear over the full range of the assay described below. Make sure to use only the linear portion of the curve to assess the concentration of your libraries.

3.3.1 Standard curve

1. Thaw the DNA standard provided with the PicoGreen reagent (100 ng/µl)
2. Label eight 1.5 ml microcentrifuge tubes 1 – 8, and transfer 1× TE into them as follows:
   a. Tube 1: **594 µl**
   b. Tubes 2 – 8: **300 µl**
3. Transfer **6 µl** of DNA standard to Tube 1 (100× dilution: 1 ng/µl) and vortex for **10 sec**.
4. Transfer **300 µl** from Tube 1 to Tube 2 and vortex for **10 sec**.
5. Transfer **300 µl** from Tube 2 to Tube 3 and vortex for **10 sec**.
6. Continue the dilution series by transferring **300 µl** from one tube into the next and vortexing **10 sec**, until **Tube 7**. Tube 8 constitutes the "no DNA control".

7. Transfer **100 µl** of each DNA standard dilution to the wells of column 12 of two 96-well black fluorometer plates (for duplicate measurements). The amounts of DNA per standard well are as listed in Table 2.

Tube #	Well	DNA Concentration
Tube 1	A12	100.00 ng/well
Tube 2	B12	50.00 ng/well
Tube 3	C12	25.00 ng/well
Tube 4	D12	12.50 ng/well
Tube 5	E12	6.25 ng/well
Tube 6	F12	3.13 ng/well
Tube 7	G12	1.56 ng/well
Tube 8	H12	0.00 ng/well

Table 2: DNA concentration of the 8-point standard curve for the fluorometric assay of Amplicon libraries.

3.3.2 Assay

1. Transfer **99 µl** of 1× TE Buffer to the remaining 88 wells (or as needed) of each of the 96-well black fluorometer plates.
2. Transfer **1.0 µl** of each purified Amplicon DNA sample (from Section 3.2.1 or 3.2.2) to the appropriate wells of the fluorometer plates. Make sure to use a fresh tip for each sample.
3. Mix by pipetting up and down 4 times, using a multichannel pipettor set to 100 µl. Again, make sure to use a fresh tip for each sample.
4. Carry out the assay as described by the manufacturer of the Quant-iT PicoGreen dsDNA Assay Kit, adding 100 µl of a 1:200 dilution of PicoGreen reagent to each well. Mix carefully by pipetting up and down 4 times. Use a fresh tip for each well.
5. Verify that the R^2 value of the standard curve is at least 0.98.
   a. If it is not, check if the top point of the curve is below the curve; your fluorometer may not have a sufficient dynamic range for the top point. If that is the case, eliminate the top point and recalculate.
   b. If the R^2 value is below 0.98 and it is not due to a dynamic range issue, repeat the assay, pipetting carefully. See an example standard curve in Appendix, Section 4.3.
6. Verify that the sample readings fall within the range of the standard curve.
   a. If any sample readings exceed the highest standard curve value, dilute and re-measure these samples, and take the additional dilution factor into account when calculating final concentration.
   b. If any sample readings fall below 5 ng/ul, it is recommended to verify the size and purity of the Amplicon before proceeding. If necessary, repeat the purification or preparation of these Amplicons.

3.3.3 Amplicon Dilution and Pooling

1. Calculate the concentration of each Amplicon in molecules/µl, using the following equation:

$$\text{Molecules/µl} = \frac{\text{sample conc [ng/µl]} \times 6.022 \times 10^{23}}{656.6 \times 10^{9} \times \text{amplicon length [bp]}}$$

2. Dilute each Amplicon (separately) to 1×10^{9} molecules/µl, in 1× TE Buffer. This is easily done by adding 1 µl of each Amplicon sample in the volume of TE calculated as follows:

$$\left(\frac{\text{molecules/µl (from step 1)}}{10^{9}} - 1 \right) \text{µl}$$

3. If multiple Amplicons are to be sequenced together, *i.e.* within a region of a PicoTiterPlate device (which is typical), mix an equal volume (*e.g.* 10 µl) of each of these diluted Amplicons to prepare Amplicon pools.

4. Dilute each Amplicon pool to 10^{7} molecules/µl by adding 2 µl of the Amplicon pool from step 3 to 198 µl Molecular Biology Grade Water. Store the 1×10^{9} molecules/µl stock and the diluted aliquots at -20°C.

4. Appendix

4.1 PCR / Primers Optimization

Amplification of any given target sequence may require individual testing and optimization. Addition of the adaptors and MIDs defined in the GS FLX Titanium chemistry may introduce additional possibilities for primer duplex and hairpin formation, and it is recommended to test these both in a prediction algorithm such as that hosted by Integrated DNA Technologies (IDT; see at **http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/**) and by experimentation.

The PCR conditions given in this method are a suggested starting point but will not be optimal for every Amplicon. The method uses the Roche FastStart High Fidelity Polymerase, which in our hands has routinely provided robust amplification of fragments in the 200 – 600 bp size range from a variety of targets with variable %GC content. Be sure to include appropriate positive and negative controls in your optimization tests.

The optimal annealing temperature can be predicted from the melting temperature of the gene-specific part of the fusion primers for each target sequence, but, again, empirical optimization may be necessary.

4.2 PCR Plate Layout

It is convenient to use only columns 1 – 11 of a 96-well plate when preparing and purifying your Amplicons, for a total of 88 Amplicons per plate. This way, you can use the same plate layout for the quantitation assay (where column 12 is used for the standard curve) as for the amplification and the library purification, minimizing the risk of confusion in the identity of the Amplicons.

4.3 Example Library Quantitation Standard Curve

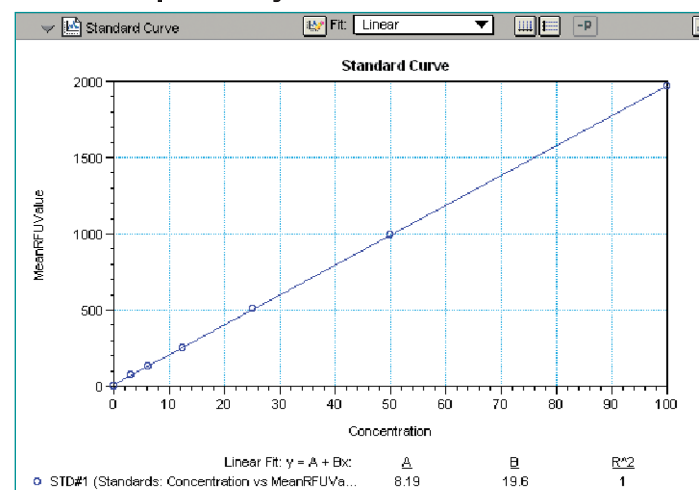


Figure 2

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