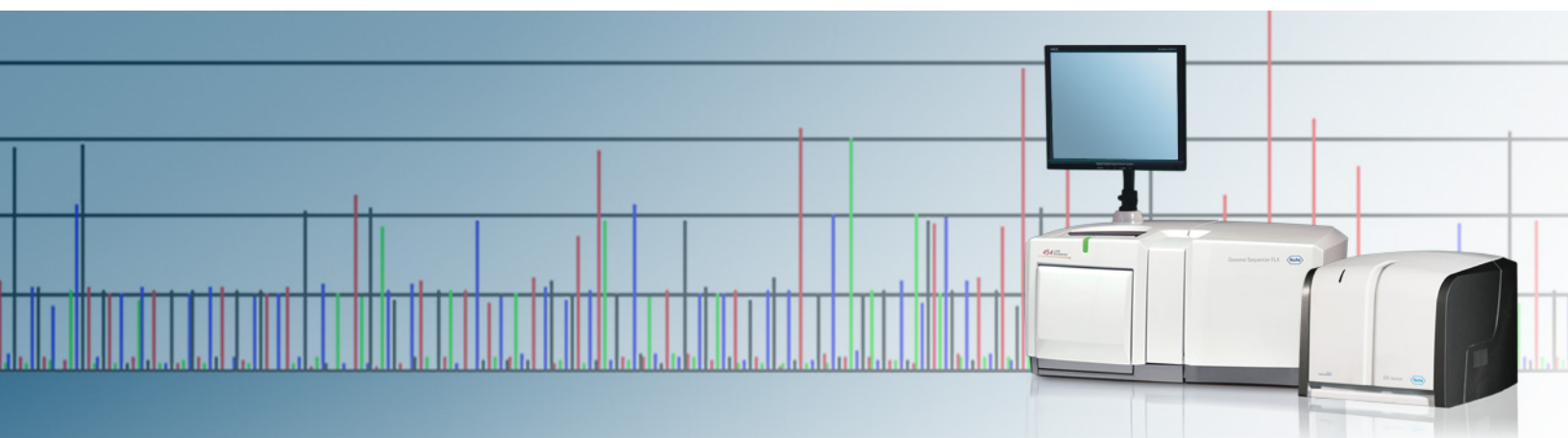




TCB No. 2011-001

February 2011

Technical Bulletin

GS FLX System

Amplicon Sequencing with Various emPCR Amplification Conditions

Summary

Consideration of amplicon length is critical to the design of a successful sequencing experiment. Additionally, the method used to prepare amplicon library beads for sequencing is equally important. The purpose of this Technical Bulletin is to first, demonstrate how amplicon length can affect the amount of high quality reads generated per sequencing run; second, offer recommendations on sequencing performance of amplicons of varying length under three different emPCR amplification protocols:

1. Short Fragment Lib-A emPCR
2. GS FLX Titanium Lib-A emPCR
3. Long Fragment Lib-A emPCR

Effect of amplicon length on number of reads

For *GS FLX Titanium Sequencing*, we recommend that amplicon targets be designed between 200 and 600 bp (including fusion primers and MID sequences) to generate high quality sequences based on TCB No. 013-2009. As a general rule, both solution and solid-phase PCR (emPCR amplification) favor the amplification of shorter fragments. Figure 1 shows the results of four different sized amplicon pools prepared for sequencing using the emPCR amplification protocol in the *GS FLX Titanium Lib-A emPCR* method manual.

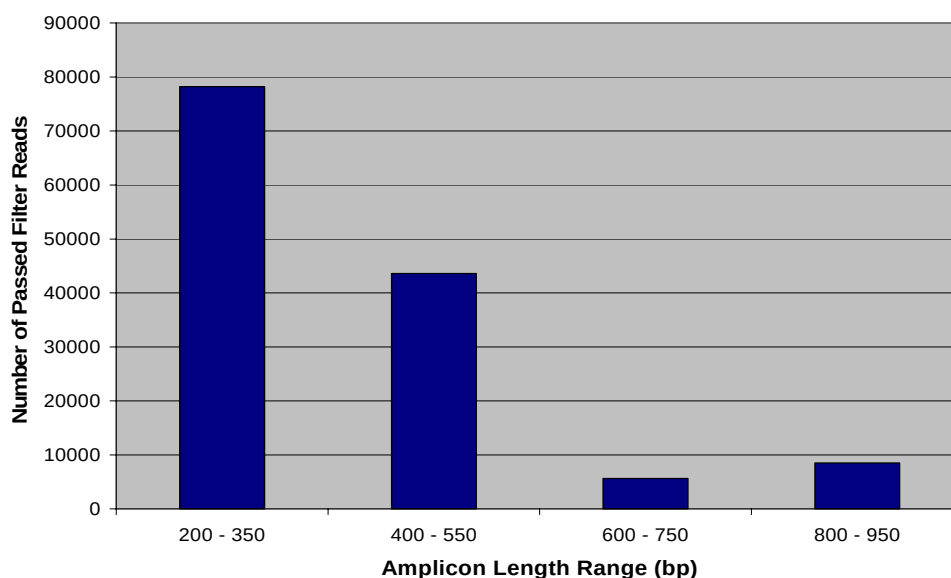


Figure 1: Effect of Amplicon Length on Number of Passed Filter Reads. The amplicon pools are derived from individual amplicons designed every 50 bp.

The data show the expected inverse relationship where as amplicon length increases the number of Passed Filter Reads decreases. Therefore, when possible, amplicons should be designed as short as possible to maximize the number of Passed Filter Reads based on the specific experimental design requirements.

Effect of emPCR amplification chemistry on amplicon sequencing quality

If the design of amplicon targets cannot be modified to accommodate for the length recommendations (200-600 bp), modifications to the *GS FLX Titanium Series* emPCR amplification protocol can be assessed. Modifications to adjust the efficiency of emPCR amplification can optimize sequencing throughput. Presented in this section are two emPCR amplification modifications (in addition to *GS FLX Titanium* emPCR amplification) and their effect when sequencing amplicons whose lengths are 200 to 950 bp in 50 bp increments.

Short Fragment Lib-A emPCR Amplification

Amplicons that are 200 bp or less are considered short fragments. Application Brief No. 001-2010, Section 2, describes a modification to the standard amplicon emPCR amplification protocol outlined in the *emPCR Method Manual* for the *GS FLX Titanium emPCR Kit (Lib-A)*.

To set up short fragment emPCR amplification, two modifications to the Live Amplification Mix preparation are made. First, the amount of Amplification Primer A and B used is decreased, such that 25% of the original volume is used. To compensate for this loss in volume, the amount of Molecular Biology Grade water is increased. An example using only Amplification Primer A is shown in Table 1.

Reagent	Volume (µl)	Volume (µl)
	16 Tubes	16 Tubes
	GS FLX Titanium emPCR	Short Fragment emPCR
Mol. Bio. Grade Water	1200	1372.5
emPCR Additive	1500	1500
5x Amplification Mix	780	780
Amplification Primer A	230	57.5
emPCR Enzyme Mix	200	200
PPiase	5	5
Total:	3915	3915

Table 1: Preparation of small volume Live Amplification Mix for short fragments and standard GS FLX Titanium emPCR amplification. Modified volumes are bolded.

Only a modification to the Live Amplification Mix is necessary for short fragment emPCR amplification. The rest of the protocol follows the *emPCR Method Manual* for the *GS FLX Titanium emPCR Kit (Lib-A)*.

Long Fragment Lib-A emPCR Amplification

Amplicons that are 550 bp or greater are considered long fragments and can benefit from the following recommendation. A modification to the emPCR amplification Live Amplification Mix preparation and to the thermocycling conditions will provide for more efficient amplification of longer fragments. The rest of the protocol follows the *emPCR Method Manual* for the *GS FLX Titanium emPCR Kit (Lib-A)*.

Reagent	Volume (μl)
	16 Tubes
	Long fragment emPCR
Mol. Bio. Grade Water	1050
emPCR Additive	1500
5x Amplification Mix	860
Amplification Primer A	300
emPCR Enzyme Mix	200
PPiase	5
Total:	3915

Table 2: Preparation of small volume Live Amplification Mix for long fragments. Modified volumes are bolded.

Table 2 shows the modification for both the 5x Amplification Mix and Amplification Primer, which increase in volume (to compensate, the water volume decreases). The following thermocycling conditions also need to be used:

1x	4 minutes at 94°C
50x	30 seconds at 94°C, 10 minutes at 60°C
1x	10°C on hold

Amplicon Sequencing Quality versus Length

To demonstrate the effect of both short and long fragments and of the *GS FLX Titanium emPCR (Lib-A)* emPCR amplification conditions on the sequencing quality of various amplicons, four amplicon pools were created. Each pool was amplified using the three afore mentioned emPCR amplification protocols. The results are shown in Figure 2.

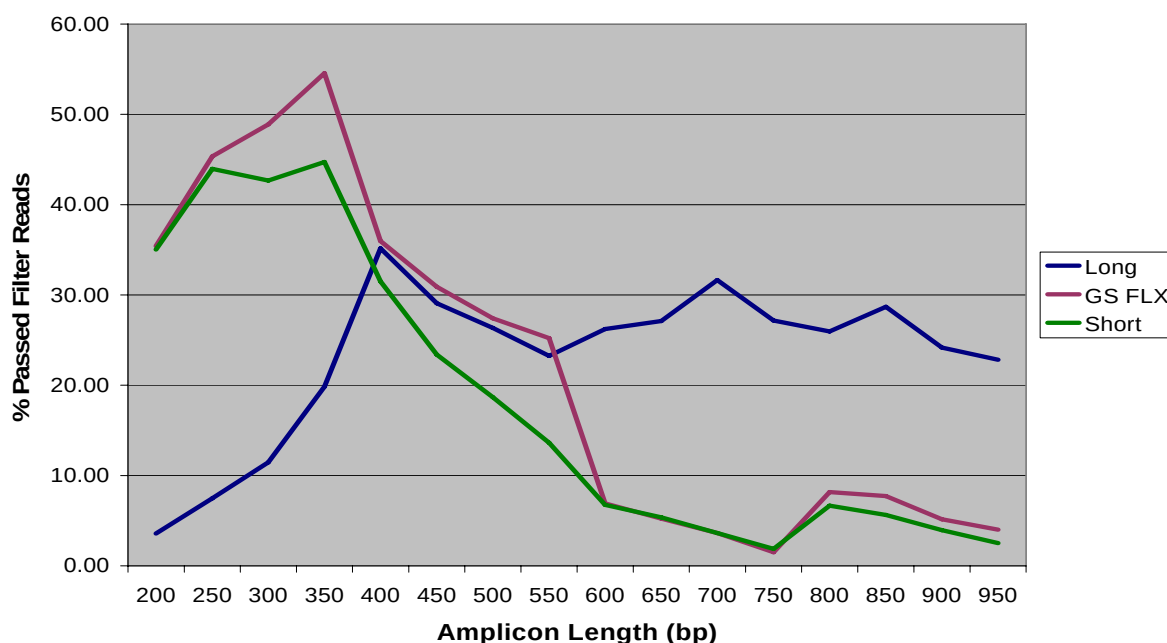


Figure 2: Amplicon sequencing under various emPCR amplification conditions

The designed amplicon length will help determine the optimal emPCR amplification protocol. Amplicons that are 400 bp or less should not be amplified with the long fragment emPCR amplification modifications. This will result in too many targets can be amplified onto the DNA Capture Bead resulting in high incompleteness efficiency, thus reducing the quality and total throughput at sequencing. Alternatively, amplicons that are 500 bp or longer should be amplified following the long fragment emPCR modification as this will result in the highest number of amplified targets on the DNA Capture Bead without the concern for generating too many amplified targets. Table 3 summarizes the recommendations:

Amplicon Length	Recommendation
200 bp or less*	Short fragment emPCR*
200 – 550 bp	GS FLX Titanium
550 bp or longer	Long fragment emPCR

Table 3: Recommendations for emPCR amplification modifications based on amplification length

*Recommendation from the Application Brief No. 001-2010

Conclusions

When possible, amplicons should be designed shorter than 500 bp when using the *GS FLX Titanium Sequencing Kit XLR70* since the longest expected read length of a balanced genome is approximately 500 bp with 200 cycles. In general, shorter amplicons generate a greater number of high quality reads. If experimental design constraints do not allow for such amplicon length designs, modifications to the emPCR amplification protocol need to be considered.

For Life Science Research Only. Not for use in Diagnostic Procedures.

454, 454 SEQUENCING, GS FLX TITANIUM, GS JUNIOR, EMPCR and PICOTITERPLATE are trademarks of Roche

Other brands or product names are trademarks of their respective holders