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# Technical Bulletin

## Genome Sequencer FLX System

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### Amplicon Fusion Primer Design Guidelines for GS FLX Titanium Series Lib-A Chemistry

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#### Summary

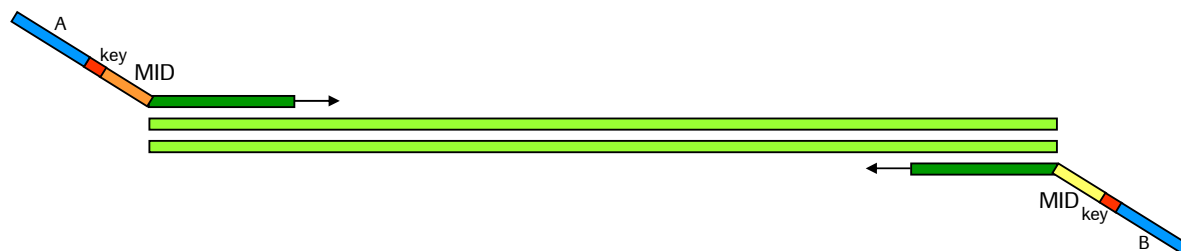
In the 4th Quarter of 2009, new GS FLX Titanium series protocols and reagents (Lib-A chemistry) for amplicon library sequencing will be available. This Technical Bulletin provides guidelines for fusion primer design and generation of amplicon libraries for this new chemistry in advance of its release, allowing users to take advantage of improvements sooner. Included in these improvements are:

- Sequencing of longer amplicon designs
- Significant increase in reads per run
- Improved read representation in bidirectional sequencing
- Simplified reagent kit choices and protocols

Also, GS FLX Titanium series kits and protocols will allow the possibility of sequencing existing amplicon libraries created using the GS FLX Standard series adaptor design – although it's highly recommended to transition amplicon library designs to the new GS FLX Titanium series format, for optimal performance.

## Guidelines for Amplicon Design

Amplicon Fusion Primers must contain a directional **GS FLX Titanium Primer A** or **Primer B** sequence (which includes a four-base library “**key**” sequence) at the 5-prime portion of the oligonucleotide in addition to the **template-specific** sequence at the 3-prime end. An optional **Multiplex Identifier (MID)** sequence may be added between the **Primer A** (or **Primer B**) and **template-specific** sequences to allow for automated software identification of samples after pooling/multiplexing and sequencing (also referred to as “barcoding”):



The nucleotide sequences of the Titanium Fusion Primers (including the key, which is underlined and highlighted in red) are as follows. This is followed by an MID (optionally) and your template-specific sequence:

### Forward primer (Primer A-Key):

5' - **CGTATCGCCTCCCTCGCGCCA****TCAG** - {MID} - {template-specific-sequence} - 3'

### Reverse primer (Primer B-Key):

5' - **CTATGCGCCTTGCCAGCCCGC****TCAG** - {MID} - {template-specific-sequence} - 3'

We recommend designs where the total length of the amplified products (including Fusion Primers) are between 200 and 600 bp. In all cases, total amplicon length should be less than 800 bp to facilitate high quality sequencing. When possible, design amplicons to cover the sequence of interest within the first 400 bp of sequencing; i.e., the first 400 bp after the adaptor sequence but including the key and both MID sequences. Note that the template-specific parts of the amplification primers should not be used for data analysis, as they will not reflect the actual sequence of the target sequence. The GS Amplicon Variant Analysis (AVA) software automatically trims this portion of the amplicon read during alignment to the user-provided reference sequence.

## Multiplexing

There are many methods to segregate samples to maximize the throughput from a single sequencing run. These include separating the samples physically (loading samples in different regions of the Pico Titer Plate (PTP) gasket), coded separation using multiplex identifiers (MIDs), or a combination of the two. If employed, MID sequences should be used in both the A and B Fusion Primers. Using different MIDs in each of the two Fusion Primers will enable a broad range of multiplexing possibilities – up to 196-fold with 14 MIDs on each end. In all cases, bidirectional sequencing should be employed.

While other barcode sequences may be incorporated, we recommend using MIDs from the Standard 454 set in the table below or from other 454 documents including Technical Bulletins. These 10-mer sequences have been carefully engineered to avoid mis-assignment of reads and are tolerant to several errors, such as those often introduced during primer synthesis.

For your convenience, these 14 MID sequences have been pre-loaded in the Amplicon Variant Analyzer (AVA) Software developed by 454 to analyze data from Amplicon library sequencing:

ID	MID Sequence	ID	MID Sequence
MID1	ACGAGTGCCT	MID8	CTCGCGTGTC
MID2	ACGCTCGACA	MID9	TAGTATCAGC
MID3	AGACGCACTC	MID10	TCTCTATGCG
MID4	AGCACTGTAG	MID11	TGATACGTCT
MID5	ATCAGACACG	MID12	TACTGAGCTA
MID6	ATATCGCGAG	MID13	CATAGTAGTG
MID7	CGTGTCTCTA	MID14	CGAGAGATAC

## Estimating Amplicon Sequencing Throughput

The table below lists the typical amplicon sequencing yield for each PTP region size (gasket type) for a sequencing run using the *GS FLX Titanium Sequencing Kit XLR70*. Comparing these numbers with the throughput requirements of your experiment will allow you to determine the multiplexing strategy to use as well as the appropriate region format. These choices then inform the emPCR format and number of emulsions to prepare.

Region Size	No. Regions	HQ Reads / Region	Cups/Tubes per Region
Large	2	360,000-520,000	MVE*: 2A + 2B or LVE: ½A and ½B or SVE: 8A + 8B
Medium	4	130,000-200,000	MVE: 1A + 1B or SVE: 4A + 4B
Med/Small	8	65,000-100,000	SVE: 2A + 2B
Small	16	20,000-30,000	SVE: 1A + 1B

\*MVE refers to a new Medium Volume Emulsion oil format that will soon be available

## Backward Compatibility

It is highly recommended to redesign amplicons in order to use all the benefits of the GS FLX Titanium series. In addition to generating an increase number of amplicons with the Titanium series, a more balanced ratio of forward and reverse reads has been observed in bidirectional sequencing. Conversely, reduced enrichment efficiency or number of reads may be observed when using the GS FLX Standard series amplicon libraries with GS FLX Titanium emPCR reagents.

Further general information on amplicon sequencing is available in the *GS FLX Amplicon DNA Library Preparation Method Manual*. While that manual was written in support of GS FLX Standard series sequencing much of the information is applicable and provides useful background.

If you have any questions on this material please contact your local Roche representative.



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