Dear Valued Customer,

In the 4th Quarter of 2009, methods and reagents will be available for sequencing of Amplicon libraries using GS FLX Titanium series reagents. So that you may prepare for the release of these products, guidelines are provided below to enable you to design Fusion Primers and generate Titanium Amplicon libraries in the interim.

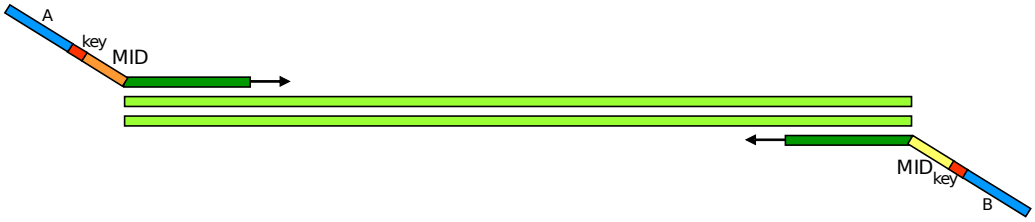
Sequencing of Amplicon libraries using Titanium series protocols provides numerous benefits:

- -Much longer amplicons may be sequenced in a single read
- -2 to 3-fold increase in reads per Run
- -Improved read representation in bidirectional sequencing

While less desirable, sequencing of existing FLX Standard series Amplicon libraries is supported on the forthcoming Titanium series protocols.

**Guidelines for Amplicon Design**

Amplification Fusion Primers must contain a directional **Titanium Primer A or B** sequence (which includes a 454 library "**key**" sequence) at the 5-prime end of the oligonucleotide in addition to the **template-specific** sequence at the 3-prime end. A **Multiplex Identifier** (MID) sequence may be added in between the Titanium Primer 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'-CGTATCGCCTCCCTCGCGCCATCAG-MID-template-specific-3'**

Reverse primer (Primer B-Key):

**5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-template-specific-3'**

We recommend designs where the total length of the amplified products (including Fusion Primers) is 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 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 in different regions in the PTP gasket), by multiplexing with 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 misassignment 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:

| | |
|---|---|
| MID1 | ACGAGTGCGT |
| MID2 | ACGCTCGACA |
| MID3 | AGACGCACTC |
| MID4 | AGCACTGTAG |
| MID5 | ATCAGACACG |
| MID6 | ATATCGCGAG |
| MID7 | CGTGTCTCTA |

| | |
|---|---|
| MID8 | CTCGCGTGTC |
| MID9 | TAGTATCAGC |
| MID10 | TCTCTATGCG |
| MID11 | TGATACGTCT |
| MID12 | TACTGAGCTA |
| MID13 | CATAGTAGTG |
| MID14 | CGAGAGATAC |

**Estimated Amplicon Sequencing throughput**

The table below lists the typical amplicon sequencing yield for each PTP Loading Region configuration 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 Loading Region format. These choices then inform the emPCR format and number of emulsions to prepare.

| **Loading Region Size** | | **HQ Reads / Region** | **Cups/Tubes per Region** |
|---|---|---|---|
| Large | 2 | 360,000-520,000 | MVE: 2A + 2B or<br>LVE ½A and ½B |
| Medium | 4 | 130,000-200,000 | MVE: 1A + 1B or<br>SVE: 4A + 4B |
| Med. Small | 8 | 65,000-100,000 | MVE: 1A + 1B or<br>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**

There are benefits to redesigning amplicons for Titanium series sequencing and this is recommended where possible to maximize performance. In addition to the ability to generate more, longer amplicons with Titanium series, a more equivalent ratio of forward and reverse reads has been observed in bidirectional sequencing. Conversely, reduced enrichment efficiency may be observed using FLX Standard series Amplicon libraries with 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 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.