Technical Bulletin

GS FLX System & GS Junior System

Multiplex Identifier (MID) Adaptors for Rapid Library Preparations

Summary

This Technical Bulletin includes information on ordering and preparing MID Adaptors, instructions for their use in the Rapid Library Preparation method, and how to configure the software to recognize them. Libraries prepared with MID Adaptors may be multiplexed during Amplification in order to facilitate sample identification following sequencing on the Genome Sequencer FLX Instrument. This enables the most efficient sequencing for the user. When using the GS FLX Titanium System, it also provides the flexibility to combine gasket format with MIDs to meet one's particular multiplexing needs.

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454 SEQUENCING

Introduction

The information contained in this document enables Users of the GS FLX Titanium chemistry to employ more than the 12 Multiplex Identifier (MID) Adaptors (numbers 1-12) that are available in the current GS Rapid Library MID Adaptors Kit (Part No. 05619211001).   Please note that the *GS FLX Titanium Rapid Library Preparation Method Manual,* the *GS Junior Titanium Rapid Library Preparation Method Manual* and the GS FLX Titanium General Library Preparation Kit can be used to prepare a library without reference to the information contained in this bulletin. You do not need to read this document when preparing standard, non-MID libraries.

Through an agreement with IDT (Integrated DNA Technologies), an additional 120 MID Adaptors (numbers 13-132) are orderable from their website at www.idtdna.com. An important difference exists between the 12 first MID Adaptors and the extended 120. The 12 first Rapid Library MID Adaptors are ready-made double-stranded sequences, whereas the extended Rapid Library MID Adaptors are delivered as two oligonucleotides (labeled RLxxx Adaptor A and RLxxx Adaptor B, where xxx is the MID adaptor number) that must be annealed into a double-strand adaptor prior to library preparation.

This document describes the preparation and use of RL MID Adaptors numbers 13-132. These may be used as an extension for the Adaptors provided in the GS FLX Titanium Rapid Library Preparation Kit. An MID Adaptor includes the MID, an 11nucleotide base pair tag, unique to each MID. When different libraries are prepared with different MID Adaptors, they are amplified during emPCR amplification and sequenced together in a multiplex fashion; the sequencing reads can be analyzed by the data analysis software after the sequencing Run, such that the reads from each of the pooled libraries are categorized by their MID tags and correctly assigned.

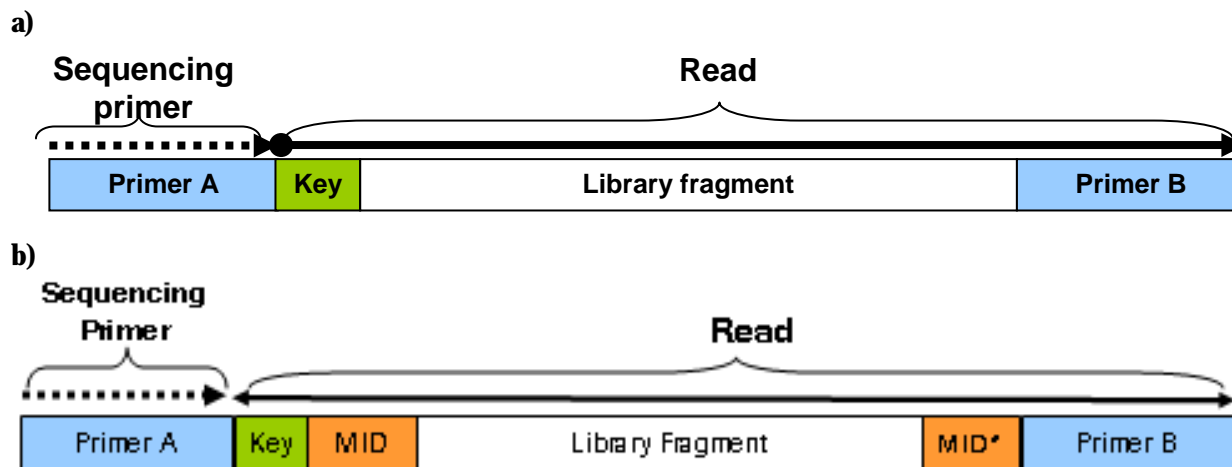Figure 1 illustrates the structure of a completed library DNA fragment with and without an MID Adaptor:

a)

Sequencing primer

Read

| Primer A | Key | Library fragment | Primer B |

b)

Sequencing Primer

Read

| Primer A | Key | MID | Library Fragment | MID* | Primer B |

Figure 1: Schematic of final library constructs with non-MID Rapid Library Adaptors (a) and with MID Adaptors (b)
* modified version of reverse complemented MID

The MID tag is added to the 3' end of Adaptor A, immediately following the four-base Key. Therefore, Adaptor A must be synthesized with the desired MID sequence. Additionally, due to the adaptor design for Rapid libraries, a modified version of the reverse complemented MID is incorporated into the sequence of Adaptor B. The Rapid Library adaptor is a double strand sequence, ligated to both ends of the library fragment. The 'top' strand contains Primer A, while the 'bottom' strand contains Primer B.

Multiplex Identifier Adaptor Design

A robust set of 132 eleven base Multiplex Identifier (MID) sequences was designed to facilitate library multiplexing in the 454 Sequencing System. A length of eleven nucleotide bases is sufficient to ascertain that, for the large number of reads involved and the design parameters considered, the chances of mis-assigning reads is extremely low. Different MID-labeled libraries can be subsequently mixed during emPCR amplification and sequencing, and the resulting library reads can be sorted after sequencing using SFF software tools as outlined in the *Genome Sequencer Data Analysis Software Manual* (for the GS FLX Titanium Series) or the *454 Sequencing System Software Manual, v 2.5* (for the GS Junior Titanium Series).

Rapid Libraries can be prepared using MID Adaptors instead of the Adaptors supplied in the GS FLX Titanium Rapid Library Preparation Kit. When used as a set, the MIDs allow extensive error correction capability as well as robustness to avoid read mis-assignment. Error is defined as a single insertion, deletion, or substitution.

The MIDs were designed so they:

- Use at most 21 flow cycles
- Are all of the same length
- Avoid nucleotide repeats
- Do not form a repeat with last base of the Key sequence (T)
- Are robust to possible errors during primer synthesis and/or sequencing to prevent read mis-assignment.
- Must be used with an overhanging T present in the "top" oligo

One critical element of the design is robustness against deletions, which are common during oligonucleotide synthesis. For example, two sequences like ACTGA and CTGAG may seem far apart when considering substitutions , but they can become one base apart or even identical with only a single deletion of the 5'A of ACTGA.

The first set of the twelve Rapid Library MIDs is shown in Table 1. These MIDs have been included in the MIDConfig.parse file described in the *Genome Sequencer Data Analysis Software Manual*, section 13.4.15 (for the GS FLX Titanium Series) or the *454 Sequencing System Software Manual, v 2.5* Part C, section 4.6.2 (for the GS Junior Titanium Series).

MID	Sequence
1	ACACGACGACT
2	ACACGTAGTAT
3	ACACTACTCGT
4	ACGACACGTAT
5	ACGAGTAGACT
6	ACGCGTCTAGT
7	ACGTACACACT
8	ACGTACTGTGT
9	ACGTAGATCGT
10	ACTACGTCTCT
11	ACTATACGAGT
12	ACTCGCGTCGT

Table 1: List of MID sequences (numbers 1-12)

The list of the extended 120, Rapid Library MIDs, is available on the IDT website. These MID sequences are NOT included in the MIDConfig.parse file. However, the MIDConfig.parse file will need to be updated to reflect the entire set of MIDs used to create the multiplexed library pool. See the section "Configuring the DataAnalysis Software".

Note: While the Extended MID Set has been carefully designed to reduce the chance of mis-assigning reads, not all sequences have yet been thoroughly tested in library construction. It is possible that one or more MIDs on the list may underperform. In particular, our experience to date with MIDs has shown that approximately one in ten sequences performs below expectations, because of unexpected dimerization/ligation events or unanticipated PCR amplification artifacts.

Obtaining and Preparing MID Adaptors

Ordering Oligonucleotides

Note: The oligonucleotides required for adaptor annealing may be ordered from IDT. Screenshots provided herein were obtained from IDT (http://www.idtdna.com) and are meant to serve as an example of the process one would be expected to follow.

1. For each different MID desired you must obtain the required oligonucleotides and prepare a new MID Adaptor mix. Each MID Adaptor mix is comprised of a Top Adaptor (RLxxx Adaptor A) and a Bottom Adaptor (RLxxx Adaptor B) that are annealed in an equimolar ratio as described in the next section. Figure 2 is a screenshot of the introductory webpage for the ordering of 454 Sequencing MIDs. From this page, one is directed to the webpage for the ordering of Rapid Library MIDs, as in Figure 3.

Figure 2: Overview page of the IDT website introducing the Rapid Library MIDs as well as the Fusion Primer MIDs for Amplicon Sequencing

Figure 3: Screenshot of the Rapid Library MID Adaptors order webpage. Shown is a subset (numbers 43-57) of the Rapid Library MIDs orderable from IDT. The first set of 12 MIDs Adaptors is available from Roche, while the extended set of MIDs (numbers 13-132) is available from IDT

2. Oligonucleotides are shipped lyophilized (dry). Store these materials per the supplier's recommendations. Generally, -15°C to -20°C storage is appropriate for lyophilized material.

Purification: All oligonucleotides must be ordered with HPLC purification.

Annealing Adaptors

Each MID Adaptor mix (mixture of RLxxx Adaptor A and RLxxx Adaptor B) must be prepared individually. The instructions below outline the process of making one such MID Adaptors mix.

1. Obtain or prepare 1x TE Buffer (10mM Tris, pH 8.0, 1 mM EDTA)

    49.5 ml Molecular Biology Grade Water (many possible)
     0.5 ml 100× TE, pH 8.0   (*e.g.* Fisher Scientific cat. number BP1338-1)
    50.0 ml total

    a. Combine the above ingredients and mix thoroughly.
    b. Store at –15 to –25ºC (or temporarily at $+2^0$ to $+8^0$ C).

2. Prepare Oligo Buffer (1x TE and 50 mM NaCl) as follows:

    9.9 ml 1x TE, pH 8.0   (recipe above)
    0.1ml 5 M NaCl   (*e.g.* Invitrogen cat. number 24740-011)
    10 ml total

    a. Combine the above ingredients and mix thoroughly.
    b. Store at –15 to –25ºC (or temporarily at $+2^0$ to $+8^0$ C).

3. For lyophilized (dry) oligonucleotides, briefly centrifuge the tubes to ensure that any powdered material is concentrated in the bottom of the tubes.

4. Dissolve each lyophilized oligonucleotide to a concentration of 100 µM with Oligo Buffer. Vortex to mix. Incubate at room temperature for five minutes before proceeding.

5. (Optional) Verify the concentration of the oligonucleotide stock solution via OD_{260} spectrophotometry or fluorometry. Guidance on this process can be found on the IDT website (link below) or from your oligonucleotide supplier of choice:

    http://idtdna.com/Support/Technical/TechnicalBulletinPDF/Oligonucleotide_Yield_Resuspension_and_Storage.pdf

6. Prepare the MID Adaptor by combining the complementary oligonucleotides RLxxx Adaptor A and RLxxx Adaptor B in Oligo Buffer. Add the resuspended oligonucleotides and Oligo Buffer into 0.2 ml tubes to make the MID Adaptor annealing reactions for the MID Adaptors being prepared. The final concentration of each MID Adaptor in this mix is 25 µM:

    MID Adaptor Mix

    50 µl Oligo Annealing Buffer
    25 µl RLxxx Adaptor A (100 µM)
    25 µl RLxxx Adaptor B (100 µM)
    100 µl total

7. Mix by vortexing, and spin down briefly. Place the 0.2 ml tubes in a thermocycler and run the following annealing program:
   a. 95°C for 1 minute
   b. Decrease temperature by 0.1°C / second to 15°C final temperature
   c. 14°C forever

8. The MID Adaptors will be used as a substitute for the Adaptors supplied in the GS FLX Titanium Rapid Library Preparation Kit, as described in section 3.4 (Adaptor Ligation) of the *GS FLX Titanium Rapid Library Preparation Method Manual*or the *GS Junior Titanium Rapid Library Preparation Method Manua).* The adaptor ligation step requires 1 μl of the final MID Adaptors per library, therefore enough MID Adaptors has been prepared to create ~100 libraries following the above protocol.

9. Distribute 10 μl aliquots of the MID Adaptors into new tubes for storage. Each aliquot will be sufficient for up to 10 libraries.

10. Store the MID Adaptors aliquots and any remaining separate Adaptor stocks at -15 to -25°C. These materials may be stored under these conditions for up to one year.

Configuring the Data Analysis Software

In order for the software to recognize the extended Rapid Library MIDs (or any subset of them), the MIDConfig.parse file must contain the definitions of all MIDs included in the reads. By default, MIDConfig.parse contains definitions for the first 12 Rapid Library MID Adapters only, as shown in the following block:

```
RLMIDs
{
    mid = "RL1", "ACACGACGACT", 1, "AGTCGTGGTGT";
    mid = "RL2", "ACACGTAGTAT", 1, "ATACTAGGTGT";
    mid = "RL3", "ACACTACTCGT", 1, "ACGAGTGGTGT";
    mid = "RL4", "ACGACACGTAT", 1, "ATACGTGGCGT";
    mid = "RL5", "ACGAGTAGACT", 1, "AGTCTACGCGT";
    mid = "RL6", "ACGCGTCTAGT", 1, "ACTAGAGGCGT";
    mid = "RL7", "ACGTACACACT", 1, "AGTGTGTGCGT";
    mid = "RL8", "ACGTACTGTGT", 1, "ACACAGTGCGT";
    mid = "RL9", "ACGTAGATCGT", 1, "ACGATCTGCGT";
    mid = "RL10", "ACTACGTCTCT", 1, "AGAGACGGAGT";
    mid = "RL11", "ACTATACGAGT", 1, "ACTCGTAGAGT";
    mid = "RL12", "ACTCGCGTCGT", 1, "ACGACGGGAGT";
}
```

MIDConfig.parse needs to be updated to reflect whatever the actual constellation of RL MIDs is for a particular dataset. This is done by adding a separate block for each additional constellation of MIDs that are in use, and explicitly specifying that MID scheme in the software. It is generally not appropriate to update MIDConfig.parse to use the entire 132 MID set, because that would increase the likelihood that a read with a sequencing error in the MID part of the adapter may be erroneously assigned to a MID that is not included in the experiment but happens to be the same number of errors away from the read as the correct MID. Therefore, as more experiments are done with various combinations of MIDs, this file will continue to grow, but the advantages are that it is self-documenting in terms of the different MID pools that have been used, and that it provides flexibility for the DataAnalysis applications to properly de-multiplex all of the pools without having to repeatedly update the contents of the existing block or create separate MIDConfig.parse files for each different MID pool that has been used. An alternative to adding new blocks of MID information to MIDConfig.parse would be to create additional MIDConfig.parse files and use the –mcf option in the software. It is not the recommended strategy however, because the –mcf option is supported only in the command-line versions of the assembler and mapper, not in the GUI versions.

Extended RL MID Sequences

For convenience, a MIDConfig.parse block (MID scheme) is provided that contains the entire set of 132 RL MIDs. As mentioned in the previous section, in practice additional blocks should be added that reflect only the MIDs present in the particular experiment. The following comprehensive block can be used as a template, where all the MIDs that are not used can be deleted and the title of the block (i.e. name of the MID scheme) can be customized. For a more comprehensive discussion of how to specify individual MIDs in the mapper, assembler, and sfffile utility, see the 454 Sequencing System Software Manual, v 2.3 or v 2.5, particularly section 4.6 which deals directly with GS FLX Rapid Library MIDs.

RLMIDs_CUSTOM_1
{
    mid = "RL1", "ACACGACGACT", 1, "AGTCGTGGTGT";
    mid = "RL2", "ACACGTAGTAT", 1, "ATACTAGGTGT";
    mid = "RL3", "ACACTACTCGT", 1, "ACGAGTGGTGT";
    mid = "RL4", "ACGACACGTAT", 1, "ATACGTGGCGT";
    mid = "RL5", "ACGAGTAGACT", 1, "AGTCTACGCGT";
    mid = "RL6", "ACGCGTCTAGT", 1, "ACTAGAGGCGT";
    mid = "RL7", "ACGTACACACT", 1, "AGTGTGTGCGT";
    mid = "RL8", "ACGTACTGTGT", 1, "ACACAGTGCGT";
    mid = "RL9", "ACGTAGATCGT", 1, "ACGATCTGCGT";
    mid = "RL10", "ACTACGTCTCT", 1, "AGAGACGGAGT";
    mid = "RL11", "ACTATACGAGT", 1, "ACTCGTAGAGT";
    mid = "RL12", "ACTCGCGTCGT", 1, "ACGACGGGAGT";

mid = "RL13", "AGACTCGACGT", 1, "ACGTCGGGTCT";
mid = "RL14", "AGTACGAGAGT", 1, "ACTCTCGGACT";
mid = "RL15", "AGTACTACTAT", 1, "ATAGTAGGACT";
mid = "RL16", "AGTAGACGTCT", 1, "AGACGTCGACT";
mid = "RL17", "AGTCGTACACT", 1, "AGTGTAGGACT";
mid = "RL18", "AGTGTAGTAGT", 1, "ACTACTAGACT";
mid = "RL19", "ATAGTATACGT", 1, "ACGTATAGTAT";
mid = "RL20", "CAGTACGTACT", 1, "AGTACGTGCTG";
mid = "RL21", "CGACGACGCGT", 1, "ACGCGTGGTCG";
mid = "RL22", "CGACGAGTACT", 1, "AGTACTGGTCG";
mid = "RL23", "CGATACTACGT", 1, "ACGTAGTGTCG";
mid = "RL24", "CGTACGTCGAT", 1, "ATCGACGGACG";
mid = "RL25", "CTACTCGTAGT", 1, "ACTACGGGTAG";
mid = "RL26", "GTACAGTACGT", 1, "ACGTACGGTAC";
mid = "RL27", "GTCGTACGTAT", 1, "ATACGTAGGAC";
mid = "RL28", "GTGTACGACGT", 1, "ACGTCGTGCAC";
mid = "RL29", "ACACAGTGAGT", 1, "ACTCACGGTGT";
mid = "RL30", "ACACTCATACT", 1, "AGTATGGGTGT";
mid = "RL31", "ACAGACAGCGT", 1, "ACGCTGTGTGT";
mid = "RL32", "ACAGACTATAT", 1, "ATATAGTGTGT";
mid = "RL33", "ACAGAGACTCT", 1, "AGAGTCTGTGT";
mid = "RL34", "ACAGCTCGTGT", 1, "ACACGAGGTGT";
mid = "RL35", "ACAGTGTCGAT", 1, "ATCGACAGTGT";
mid = "RL36", "ACGAGCGCGCT", 1, "AGCGCGCGCGT";
mid = "RL37", "ACGATGAGTGT", 1, "ACACTCAGCGT";
mid = "RL38", "ACGCGAGAGAT", 1, "ATCTCTGGCGT";
mid = "RL39", "ACGCTCTCTCT", 1, "AGAGAGGGCGT";
mid = "RL40", "ACGTCGCTGAT", 1, "ATCAGCGGCGT";
mid = "RL41", "ACGTCTAGCAT", 1, "ATGCTAGGCGT";
mid = "RL42", "ACTAGTGATAT", 1, "ATATCACGAGT";
mid = "RL43", "ACTCACACTGT", 1, "ACAGTGGGAGT";
mid = "RL44", "ACTCACTAGCT", 1, "AGCTAGGGAGT";
mid = "RL45", "ACTCTATATAT", 1, "ATATATGGAGT";
mid = "RL46", "ACTGATCTCGT", 1, "ACGAGATGAGT";
mid = "RL47", "ACTGCTGTACT", 1, "AGTACAGGAGT";
mid = "RL48", "ACTGTAGCGCT", 1, "AGCGCTAGAGT";
mid = "RL49", "AGACACTCACT", 1, "AGTGAGGGTCT";
mid = "RL50", "AGACATATAGT", 1, "ACTATAGGTCT";
mid = "RL51", "AGACGTGATCT", 1, "AGATCAGGTCT";
mid = "RL52", "AGAGTACAGAT", 1, "ATCTGTAGTCT";
mid = "RL53", "AGAGTATCTCT", 1, "AGAGATAGTCT";
mid = "RL54", "AGATACGCTGT", 1, "ACAGCGTGTCT";
mid = "RL55", "AGATCTAGTCT", 1, "AGACTAGGTCT";

mid = "RL56", "AGCAGCGTAGT", 1, "ACTACGCGGCT";
mid = "RL57", "AGCGCACGAGT", 1, "ACTCGTGGGCT";
mid = "RL58", "AGCGTGTGCGT", 1, "ACGCACAGGCT";
mid = "RL59", "AGCTAGATACT", 1, "AGTATCTGGCT";
mid = "RL60", "AGCTGTCGACT", 1, "AGTCGACGGCT";
mid = "RL61", "AGTATGCACGT", 1, "ACGTGCAGACT";
mid = "RL62", "AGTCGCGCTAT", 1, "ATAGCGGGACT";
mid = "RL63", "AGTCTGTCTGT", 1, "ACAGACGGACT";
mid = "RL64", "ATACACACGAT", 1, "ATCGTGGGTAT";
mid = "RL65", "ATACGCGTGCT", 1, "AGCACGGGTAT";
mid = "RL66", "ATACTAGCACT", 1, "AGTGCTGGTAT";
mid = "RL67", "ATAGAGCTAGT", 1, "ACTAGCTGTAT";
mid = "RL68", "ATATAGAGTAT", 1, "ATACTCTGTAT";
mid = "RL69", "ATCGCTCACGT", 1, "ACGTGAGGGAT";
mid = "RL70", "ATCGTCAGTCT", 1, "AGACTGAGGAT";
mid = "RL71", "ATCTCTCGTAT", 1, "ATACGAGGGAT";
mid = "RL72", "ATCTGAGACGT", 1, "ACGTCTCGGAT";
mid = "RL73", "ATGCTACGTCT", 1, "AGACGTGGCAT";
mid = "RL74", "ATGTGACTACT", 1, "AGTAGTCGCAT";
mid = "RL75", "CACGAGACAGT", 1, "ACTGTCTGGTG";
mid = "RL76", "CACGCGAGTCT", 1, "AGACTCGGGTG";
mid = "RL77", "CACGCTACGAT", 1, "ATCGTAGGGTG";
mid = "RL78", "CACGTGTATAT", 1, "ATATACAGGTG";
mid = "RL79", "CACTACGATGT", 1, "ACATCGTGGTG";
mid = "RL80", "CACTATACTCT", 1, "AGAGTATGGTG";
mid = "RL81", "CAGCGTACTGT", 1, "ACAGTAGGCTG";
mid = "RL82", "CAGTCTCTAGT", 1, "ACTAGAGGCTG";
mid = "RL83", "CATAGTCGCGT", 1, "ACGCGACGATG";
mid = "RL84", "CGAGACACTAT", 1, "ATAGTGTGTCG";
mid = "RL85", "CGAGAGTGTGT", 1, "ACACACTGTCG";
mid = "RL86", "CGAGTCATCGT", 1, "ACGATGAGTCG";
mid = "RL87", "CGATCGTATAT", 1, "ATATACGGTCG";
mid = "RL88", "CGCAGTACGCT", 1, "AGCGTACGGCG";
mid = "RL89", "CGCGATCGTAT", 1, "ATACGATGGCG";
mid = "RL90", "CGCGCTATACT", 1, "AGTATAGGGCG";
mid = "RL91", "CGTACAGATAT", 1, "ATATCTGGACG";
mid = "RL92", "CGTAGCTCTCT", 1, "AGAGAGCGACG";
mid = "RL93", "CGTATAGTGCT", 1, "AGCACTAGACG";
mid = "RL94", "CGTCAGCGACT", 1, "AGTCGCGGACG";
mid = "RL95", "CGTCGCAGTGT", 1, "ACACTGGGACG";
mid = "RL96", "CGTCTCACGAT", 1, "ATCGTGGGACG";
mid = "RL97", "CGTGACTCAGT", 1, "ACTGAGTGACG";
mid = "RL98", "CTACACGCTCT", 1, "AGAGCGGGTAG";

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        mid = "RL99", "CTACGATATGT", 1, "ACATATGGTAG";
        mid = "RL100", "CTAGACAGACT", 1, "AGTCTGTGTAG";
        mid = "RL101", "CTAGTACTCAT", 1, "ATGAGTAGTAG";
        mid = "RL102", "CTATATGTCGT", 1, "ACGACATGTAG";
        mid = "RL103", "CTATCGACACT", 1, "AGTGTCGGTAG";
        mid = "RL104", "CTATGTAGAGT", 1, "ACTCTACGTAG";
        mid = "RL105", "CTCACGTACAT", 1, "ATGTACGGGAG";
        mid = "RL106", "CTCGAGTCTCT", 1, "AGAGACTGGAG";
        mid = "RL107", "CTCGTCGAGAT", 1, "ATCTCGAGGAG";
        mid = "RL108", "CTCTACAGCGT", 1, "ACGCTGTGGAG";
        mid = "RL109", "CTGTCGTGCGT", 1, "ACGCACGGCAG";
        mid = "RL110", "CTGTGACGTGT", 1, "ACACGTCGCAG";
        mid = "RL111", "GACGCTGTCGT", 1, "ACGACAGGGTC";
        mid = "RL112", "GACGTATGACT", 1, "AGTCATAGGTC";
        mid = "RL113", "GACTAGCTAGT", 1, "ACTAGCTGGTC";
        mid = "RL114", "GAGACGTCGCT", 1, "AGCGACGGCTC";
        mid = "RL115", "GAGAGAGACGT", 1, "ACGTCTCGCTC";
        mid = "RL116", "GCGTAGACTAT", 1, "ATAGTCTGCGC";
        mid = "RL117", "GCGTCGTGTCT", 1, "AGACACGGCGC";
        mid = "RL118", "GCTCTCTACGT", 1, "ACGTAGGGAGC";
        mid = "RL119", "GTACACTGTAT", 1, "ATACAGGGTAC";
        mid = "RL120", "GTACGCGACAT", 1, "ATGTCGGGTAC";
        mid = "RL121", "GTACTATAGAT", 1, "ATCTATGGTAC";
        mid = "RL122", "GTACTGAGTCT", 1, "AGACTCGGTAC";
        mid = "RL123", "GTAGCTAGCGT", 1, "ACGCTAGGTAC";
        mid = "RL124", "GTAGTCACTGT", 1, "ACAGTGAGTAC";
        mid = "RL125", "GTAGTGTCACT", 1, "AGTGACAGTAC";
        mid = "RL126", "GTATACATAGT", 1, "ACTATGTGTAC";
        mid = "RL127", "GTCATCGTCGT", 1, "ACGACGAGGAC";
        mid = "RL128", "GTCGACACGCT", 1, "AGCGTGTGGAC";
        mid = "RL129", "GTCGAGTGAGT", 1, "ACTCACTGGAC";
        mid = "RL130", "GTCTACTATCT", 1, "AGATAGTGGAC";
        mid = "RL131", "GTGTCTAGACT", 1, "AGTCTAGGCAC";
        mid = "RL132", "GTGTGTATCGT", 1, "ACGATACGCAC";
}
```

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