Next-generation sequencing library preparation

Use of the Diffinity RapidTip® in amplicon library preparations improves sequencing accuracy with Roche 454® Genome Sequencer FLX Systems

The Diffinity RapidTip method is easily adapted into the GS FLX Titanium® library purification step for effective removal of excess primers and primer-dimers prior to clonal amplification to enable high confidence base calling.

Introduction

Next-generation sequencing (NGS) systems rely on carefully prepared templates to generate accurate, high throughput data. In the Roche 454 GS FLX system, sequencing templates are prepared by emulsion-based clonal amplification of a DNA library using specialized fusion primers and capture beads. Multiplex Identifiers (MIDs) may be added to the design of the fusion primers and included during library preparation as a barcoding tool to identify specific amplicons. MID-labeled amplicons must be quantified individually and pooled for emPCR (emulsion-based clonal amplification). It is difficult to quantify amplicons if they are not separated from surplus primers in the library preparation step.

Accurate quantification and the removal of extraneous small fragments from an amplified DNA library are especially important for reliable, reproducible clonal amplification by emPCR. Underestimating or overestimating the amount of DNA can lead to very poor results. If excess primers are not removed from the library, quantification will be skewed resulting in the addition of less DNA than required to the emPCR step. Inaccurate measurement will also affect the representation of pooled amplicons. In addition, excess primers can bind to the capture beads during emPCR and compete against the desired fragments. Primer-dimers can also form, becoming excellent short templates for amplification to produce a high level of background in sequencing runs. These issues may lead to the waste of an entire sequencing experiment.

The GS FLX Library Preparation Method recommends that AMPure Beads (Beckman Coulter) be used for the purification of library amplicons. The separation of amplicons from primers and primer-dimers is determined by the ratio of AMPure bead suspension to DNA in solution. The lower the AMPure/DNA ratio, the higher the size cut-off, resulting in the removal of smaller size fragments. Determining the ideal ratio can be challenging and may require further optimization for more stringent primer and primer-dimer removal. Optimization takes time and may not translate to other DNA library preparations. Rather than adjusting the stringency of the AMPure/DNA ratio, an uncomplicated method – the Diffinity RapidTip for PCR Purification –may be added to prevent carry over of primers and primer-dimers.

The Diffinity RapidTip® is a functional pipette tip based on differential affinity technology to sequester reaction impurities from PCR products. Selective adsorption particles, pre-packed into the RapidTip provide a simplified means of removing unincorporated nucleotides, primers and primer-dimers from a PCR mixture in just one minute, in a single step. The Diffinity RapidTip may be easily adapted and applied to numerous workflows involving PCR purification.

Research and Testing Laboratory, a research and development group providing contract diagnostic and DNA sequencing services, incorporates the Diffinity RapidTip into the amplicon library preparation process of their NGS workflow for microbial diversity studies using the Roche 454® Genome Sequencer (GS) FLX and FLX+ Systems. The Diffinity RapidTip is used to remove the excess primers and primer-dimers from MID-labeled amplicon libraries. RapidTip purification is followed by further treatment with AMPure beads to assure accurate quantification of amplicons prior to emPCR.

Conclusion: Use of the Diffinity RapidTip in the amplicon library purification step has significantly improved the accuracy of sequencing results by increasing signal intensities. Improved sequencing quality is likely due to RapidTip's ability to remove extraneous small fragments from DNA libraries.

Materials

Diffinity RapidTip®, RT025-048
HotStarTaq™ Plus DNA Polymerase (Qiagen)
Amplicon Library Preparation Manual, GS FLX Titanium Series
AMPure Beads
GS FLX Titanium® MV emPCR® Kit (Lib-L)
GS FLX Titanium® Sequencing Kit
96-well plate

Instruments

Multi-channel Pipettor for use with the Diffinity RapidTip
NanoDrop 1000 Spectrophotometer
Roche 454 GS FLX or FLX+

Method

Amplicon library preparation is performed using the GS Titanium Series Amplicon Library Preparation Method Manual for use with the GS FLX Titanium emPCR (Lib-L) kit to produce bead-immobilized templates for sequencing. The Diffinity RapidTip is applied in the Library Purification step.

1. Samples for evaluating microbial diversity are obtained from bacterial, archaeal, algae or fungal sources. The samples may be derived from the environment, biological fluids, or tissues.

2. Amplicon Library Preparation by PCR:

   a. A multiplex master mix is prepared with HotStarTaq Plus DNA Polymerase and special Fusion Primers consisting of the amplicon primer, MID barcode, and a 454 linker for Titanium sequencing.

   b. Multiple reactions consisting of 24 µl of PCR master mix plus 1 µl of each desired template are dispensed into a 96-well PCR plate, sealed and placed into a thermocycler. Typical PCR cycling conditions are applied.

   c. After thermocycling, the PCR amplicons ranging from 300 bp to 900 bp in length are checked for quality and size on a FlashGel® System (Lonza).

3. Amplicon Library Purification:

   a. The pooled PCR amplicon mixture is divided into 30 µl aliquots and dispensed into wells of a 96-well plate.

   b. Diffinity RapidTips are attached to a multi-channel pipettor.

   c. The PCR amplicon mixture is aspirated directly into the Diffinity RapidTip and mixed by pipetting up and down for one minute.

   d. The purified PCR amplicons are dispensed from the Diffinity RapidTip into clean wells of the 96-well plate.

   e. PCR amplicons are re-pooled and dispensed once more for additional treatment with AMPure beads using the standard protocol described in the GS FLX Titanium Amplicon Library Preparation Manual. The amplicons are now ready to be quantified.

4. Amplicon Library Quantification

   Quantification is done either by fluorometry for ssDNA or spectrophotometry for dsDNA.

5. emPCR Amplification

   Emulsion-based clonal amplification (emPCR) of the DNA fragments from the amplicon library are prepared for sequencing using the GS FLX Titanium MV emPCR Kit and associated manual.

6. Sequencing with the GS FLX System

   Bead-immobilized templates from the emPCR process are sequenced using the appropriate GS FLX Titanium Sequencing Kit and GS FLX instrument.

Results

Sequencing results are of high quality, especially when the Diffinity RapidTip is incorporated into the library purification process. Use of the Diffinity RapidTip significantly reduces the background by removing short fragments to enable high confidence calls with the GS FLX software.

454 sequencing runs with extraneous short fragments (left) compared to more accurate runs (right)

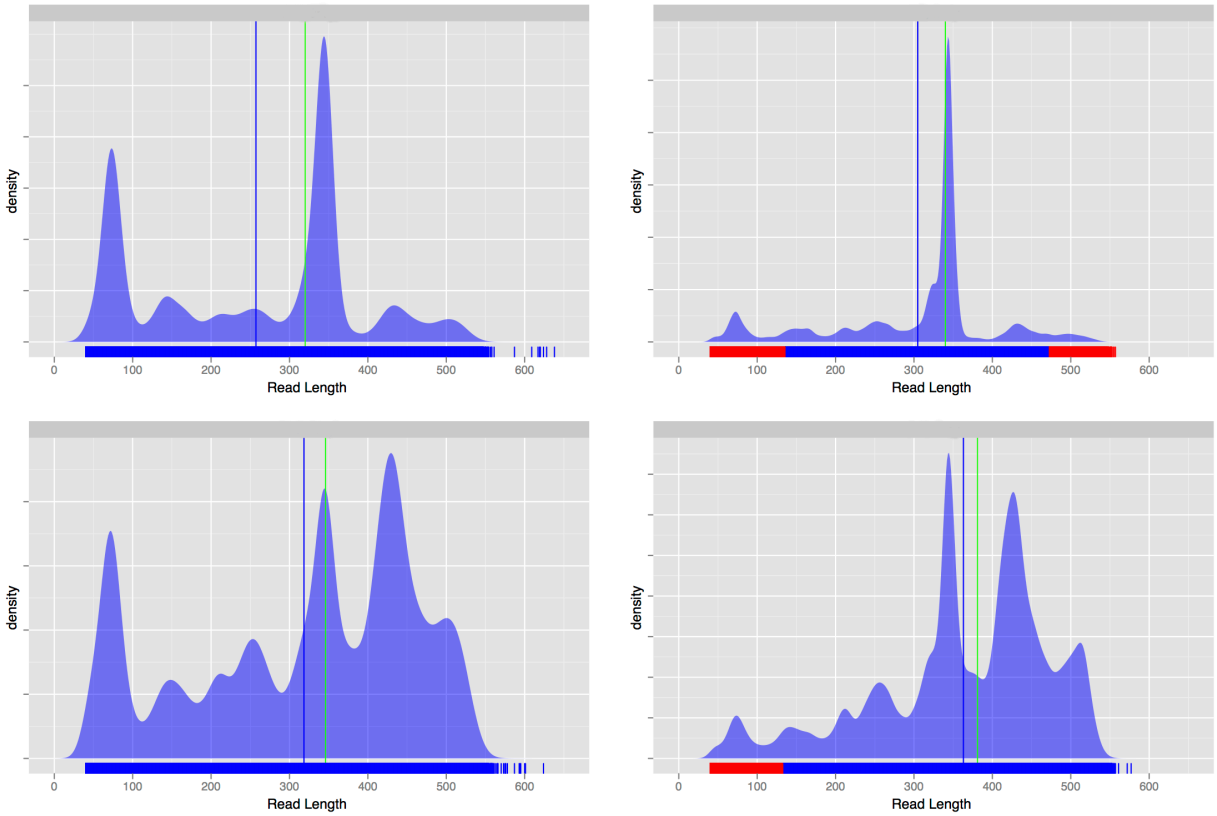

Figure 1: Read length distribution shows the difference between sequencing runs with short fragments (left) and reads with short fragments removed (right). The results show a high distribution of undesirable signal from short fragments likely caused by inefficient removal of primers and primer-dimers from amplicon libraries prior to clonal amplification by emPCR. The ambiguous signal raises the baseline, causing high background and affecting the accuracy of base calling in the sequencing results. In addition, the artifact fragments are occupying wells that cannot be used to produce meaningful data. When the short fragments are removed, the baseline is significantly decreased and base calling is more accurate. *Data supplied by Research and Testing Laboratories LLC.*

Conclusion

The Diffinity RapidTip has been incorporated into the standard protocol for microbial diversity studies at Research and Testing Laboratories for reliable sequencing accuracy with the Roche 454 GS FLX Systems. By using the Diffinity RapidTip, surplus primers and primer-dimers are effectively removed from the multiplexed amplicon library. With the majority of extraneous short fragments removed from the amplicon mixture, the AMPure beads may now be used according to the standard protocol without the need for further optimization. Templates may now be easily quantified before use in the emPCR process. By combining both the Diffinity RapidTip and the AMPure beads in consecutive purification steps, high quality NGS sequencing runs are routinely achieved.

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