Protocol adaptation for multiplexed sequencing

The following steps can be integrated into the ERRBS protocol for preparing libraries for multiplexed sequencing:

1) Follow step 1, with the following modification: at step 1.1 prepare 75 ng of high quality DNA. This protocol has not been optimized for less starting material.

2) Proceed with step 2 as detailed.

3) Proceed with step 3 as detailed.

4) Proceed with step 4 as follows:

4.1) Proceed with step 4.1 as detailed.

4.2) Proceed with step 4.2 as follows: prepare the ligation mix using barcoded methylated adapters compatible with Illumina sequencing (for example: 2 µl of TruSeq RNA Adapters) and adjust the volume of the ligation reaction mixture with water per manufacturer’s recommendations.

Note: It is critical that the barcoded adapters are methylated so they will not be converted during bisulfite treatment. It is also important to ensure that the barcoded adapters chosen for the ligation reaction in any given library are compatible with other barcoded adapters chosen for libraries planned for sequencing in the same lane. The reader is advised to carefully review the manufacturer’s guidelines for index combinations for use in the downstream sequencing planned.

4.3) Proceed with step 4.3 as detailed.

4.4) Proceed with step 4.4 as follows: purify the ligation products using a SPRI bead isolation protocol per manufacturer’s recommendations using a 1X ratio of bead volume to sample volume (50 µl of beads for the 50 µl ligation reaction). Elute into 20 µl of DNAse-free water.

5) Follow protocol 5.2 for size selection, with the following modifications:

Note: Adapters allowing for multiplexed sequencing may migrate differently from the adapters used in protocol step 5.2, and due to their structure may lead to migrations different than expected from size alone. The information below is optimized for the TruSeq adapters. The user is advised to optimize size selection parameters for the specific adapters used with the goal of obtaining insert of sizes ranging from 70 to 320 bp.

5.1) Proceed with step 5.2.5 as follows: Mark the 200 bp, 300 bp and 400 bp bands of the ladders using a razor blade or pipette tips.

5.2) Proceed with step 5.2.6 as follows: excise one slice containing the 200-300 bp and another slice containing the 300-400bp section using the marked bands as a reference. Place each of the two slices into different tubes.

6) Proceed with step 6 as detailed.

7) Perform enrichment PCR with the following modifications:

7.1) Proceed with step 7.1.1 as follows: prepare a PCR master mix containing the reagents from the table below for each 100 µl PCR reaction.

|  |  |  |
| --- | --- | --- |
| Reagent | Volume | Comment |
| 10X FastStart High Fidelity Reaction Buffer with 18 mM magnesium chloride | 10 µl |  |
| 10 mM dNTP Solution Mix | 2.5 µl |  |
| 25 µM PCR PE primer 1.0 | 2 µl | PCR PE primer 1.0: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC\*T\* = Phosphorothioate Bond |
| 25 µM PCR primer 3.0 | 2 µl | PCR primer 3.0: CAAGCAGAAGACGGCATACGAGA\*T\* = Phosphorothioate Bond |
| FastStart High Fidelity Enzyme | 1 µl | 5 units/µl FastStart Taq DNA Polymerase |
| DNase-free water | 42.5 µl |  |

7.2) Proceed with step 7.1.2 as follows: Add 60 µl of the PCR master mix to each 40 µl library fraction from step 6.3. Mix by pipetting and divide into two 50 µl PCR reactions.

7.3) Proceed with step 7.1.3 as follows: Amplify the reactions in a thermal cycler with the following protocol: Set the heat lid to 100 oC. Initialize with a step of 94 oC for 5 min. Run 18 cycles of denaturing, annealing and extension/elongation steps: 94 oC for 20 seconds followed by 60 oC for 30 seconds followed by 72 oC for 30 seconds. Run a final extension/elongation step of 72 oC for 5 min. Hold at 4 oC.

Note: 18 cycles of PCR are recommended for first time users of the protocol. The protocol can be optimized for fewer amplification cycles and the user is encouraged to optimize this step if interested.

8) Proceed with step 8 with the following modifications:

8.1) Proceed with step 8.1 as follows: Combine each 2-reaction set (per library fraction).

8.2) Proceed with step 8.2.1 as follows: Add 1.2X volume of SPRI beads (120 µl for 100 µl of PCR product) to the 200-300 bp reaction.

8.3) Proceed with step 8.2.2 as follows: Add 1.1X volume of SPRI beads (100 µl for 100 µl of PCR product) to the 300-400 bp reaction.

8.4) Proceed with protocol steps 8.2.3, 8.2.4 and 8.3 as detailed.

9) Proceed with Library Quality control as detailed in step 9.

Note: Expected concentrations are 15.9 ng/µl+-3.9 for the 200-300 bp fraction and 19.1 ng/µl +-4.3 for the 300-400 bp fraction (n=81). The average size of the libraries are 210+/-9 bp and 275+/-22 bp (n=81).

10) Prepare libraries for sequencing as specified in steps 10 through 10.3 then prepare a pool of multiplexed libraries as follows: combine the 20 µl from each barcoded 2 nM ERRBS libraries, at the desired multiplexed ratio, to obtain one multiplexed sequencing pool.

11) Proceed with protocol step 10.4 as detailed.

12) Proceed with data alignment as detailed in step 11.