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Innovating Epigenetic Solutions

Diagenode® Premium RRBS technology: cost-effective DNA methylation mapping with superior coverage

Reduced representation bisulfite sequencing (RRBS) enables genome-scale DNA methylation analysis in any vertebrate species. The assay benefits from the practical advantages of bisulfite sequencing while avoiding the cost of whole-genome sequencing. The Diagenode Premium RRBS kit makes this technology widely available and provides high coverage (up to 4 million CpGs in human samples). Multiplexing prior to bisulfite conversion allows processing of 96 samples per experiment, enabling studies of large cohorts.

DNA methylation is an essential mechanism of epigenetic gene regulation with broad relevance in development and disease. Several genome-scale methods exist for genomic DNA methylation mapping. Microarrays make it easy to study large cohorts, but they provide low genome-wide coverage, are susceptible to batch effects and are available only for the human genome. Whole-genome bisulfite sequencing provides accurate and robust coverage for most CpGs in the genome, but high sequencing costs limit its application to relatively few samples. RRBS combines the strengths of both assays, providing an accurate and cost-effective technology that is robust and flexible and can be used to study DNA methylation in any vertebrate species.

DNA methylation in vertebrates occurs mainly at CpG dinucleotides, and RRBS therefore enriches for CpG-containing DNA fragments[1,2]. To that end, the DNA is digested with the MspI restriction enzyme, which cuts DNA at C^CGG sequences regardless of the DNA methylation status at the center CpG. Small fragments are size-selected and sequenced, resulting in high coverage for CpG islands and promoter regions while also retaining significant coverage of other genomic elements such as enhancers, CpG island shores and noncoding RNAs.

The Diagenode Premium RRBS technology overcomes many limitations of the original RRBS protocol, which was time-consuming and tedious. Key strengths of the optimized protocol include (i) superior coverage of 3.5–4 million CpGs in the human genome, (ii) robustness toward batch effects, (iii) applicability to any vertebrate species and (iv) compatibility with formalin-fixed, paraffin-embedded (FFPE) samples and low-input material. Because of its versatility and high reproducibility[3], RRBS is the technology of choice for accurate DNA methylation analysis in many areas of biology and medicine.

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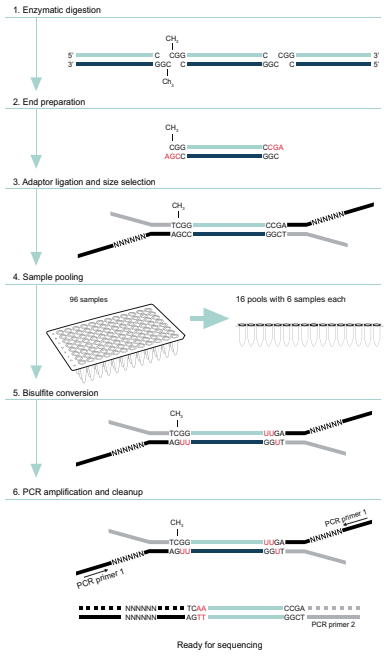

Figure 1 | RRBS workflow.

Workflow

The Diagenode Premium RRBS kit is an optimized and complete solution for RRBS containing all reagents for enzymatic digestion, bisulfite conversion and amplification.

The protocol described in **Figure 1** starts with 100 ng of DNA and has been validated for both fresh-frozen and FFPE samples without modifications. In the first step, genomic DNA is digested with MspI. This is followed by a single-tube library preparation without any intermediate cleanup steps, which minimizes material loss. The kit includes methylated and unmethylated spike-in controls to monitor bisulfite conversion efficiencies. Carefully optimized bead-based size selection retains even the smallest library fragments while removing adaptor dimers, which provides for the best sequencing results and high genomic coverage.

Table 1 | Examples of RRBS data generated with the Diagenode Premium RRBS kit using samples from four different species

Species	No. of reads aligned (alignment rate)	No. of unique CpGs covered (alignment rate)	Bisulfite conversion rate (%)	Mean sequencing depth per covered CpG (×)
Human	21,787,346 (74%)	3,913,287	99.4	14
Human FFPE	27,290,117 (79%)	2,525,053	99.7	28
Rat	27,499,313 (86%)	1,663,104	99.0	38
Dog	14,053,009 (84%)	3,572,384	99.4	9
Zebrafish	29,035,877 (83%)	1,685,466	99.3	48

One distinguishing feature of the Diagenode Premium RRBS kit is its support for early sample multiplexing prior to bisulfite conversion, which reduces the handling time and cost per sample. Custom software is provided for free on the Diagenode website to assist with optimal sample multiplexing (https://www.diagenode.com/documents/rrbs-pooling-aid). This software helps the researcher define pools of samples on the basis of their performance throughout the protocol and the compatibility of their barcodes. Each kit contains 24 different barcodes, which allows for pooling of 6–24 samples, depending on the desired sequencing depth and the size of the analyzed genome.

After multiplexing, the pooled samples are bisulfite-converted under optimized conditions that decrease DNA loss while ensuring highly efficient conversion of unmethylated cytosines. To avoid potential bias during the enrichment PCR, one should determine the optimal number of amplification cycles on the basis of the quantitative PCR results. The Diagenode MethylTaq Plus enzyme amplifies bisulfite-converted DNA with high accuracy and efficiency, which further reduces the number of PCR cycles needed. For human samples, we recommend sequencing up to six samples per lane on the Illumina® HiSeq 2000/2500 platform.

A variety of bioinformatics tools are available for analyzing RRBS data[4]. The typical analysis workflow starts with read trimming to remove adaptor sequences or low-quality bases. Subsequently, alignment and DNA methylation calling are performed using bisulfite aligners. The resulting DNA methylation data can be visualized with genome browsers like the Integrative Genomics Viewer[5] (**Fig. 2a**,**b**). Finally, the obtained DNA methylation data can be analyzed more deeply using software packages such as RnBeads[6] (for widely studied species such as human, mouse and rat) and RefFreeDMA[7] (for species lacking a high-quality reference genome).

Performance validation

The Diagenode Premium RRBS kit allowed us to generate high-quality DNA methylation profiles with excellent coverage across all vertebrate species tested. **Table 1** shows typical data obtained with human fresh-frozen and FFPE samples and with rat, dog and zebrafish samples. The genomic coverage depended on the species and exceeded 3.9 million CpGs for the human fresh-frozen sample. The bisulfite conversion rate consistently exceeded 99% in all species. A mean coverage of 10–20 per CpG should be aimed for in most cases, although certain applications work well with shallow sequencing, whereas other applications profit from deeper sequencing.

Illustrating typical results obtained with the kit, **Figure 2** shows differential DNA methylation around intergenic CpG islands in IGF2 and the unmethylated promoter region of GAPDH in two cell lines. The results were highly reproducible between replicates.

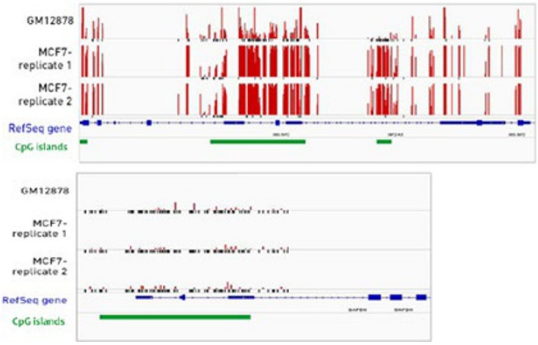

Figure 2 | DNA methylation profiles obtained with the Diagenode Premium RRBS kit. (**a**,**b**) Shown are profiles for (**a**) IGF2 and (**b**) the promoter region of GAPDH. Two human cell lines were analyzed: GM12878 and MCF7, the latter in two biological replicates. Each bar represents the percentage of DNA methylation at one CpG. The methylated CpGs are shown in red (above the horizontal lines) and the unmethylated CpGs are in black (below the lines).

Conclusion

The Diagenode Premium RRBS kit is a highly multiplexed solution for studying DNA methylation cost-efficiently in vertebrate samples, including FFPE, clinical and low-input samples. The kit contains all reagents needed to transform genomic DNA into sequencing-ready RRBS libraries with a superior coverage of 4 million CpGs.

Additional information

Please note that Diagenode also provides the RRBS technology as a service comprising library preparation, sequencing and bioinformatic analysis. For more information on Diagenode kits and services for epigenetic studies, please visit http://www.diagenode.com.

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