ZYMO RESEARCH

Controls for DNA Methylation Assays

How Controls Boost Reliability & Reproducibility

Controls For Methylation Assays: How, When & Why To Use Them

All scientific experiments need controls. Specific controls are required for certain type of experiments, such as DNA methylation assays. DNA methylation is a powerful, well-studied epigenetic mark that continues to provide extensive insight into transcription regulation, embryonic development, genome stability, and chromatin structure. Changes in DNA methylation profiles have also been linked to the development of many diseases[1]. Many clinical applications have utilized DNA methylation as a biomarker for early detection, diagnostics, and personal therapy.[2, 3] This requires accurate characterization of methylation patterns at a specific region or single-base level.

Most commonly used DNA methylation assays include bisulfite sequencing using NGS technologies, methylation-specific PCR (MSP), and methylation-sensitive restriction enzyme (MSRE) assays. To minimize bias in quantifying methylation levels, especially in clinical applications, each assay needs to be carefully designed and optimized to ensure its robustness[4]. Integrating established controls is key for optimizing assays and monitoring assay's effectiveness. For optimization of DNA methylation assays, it is best to use established DNA standards with known methylation levels. Typically, this requires a methylated and non-methylated DNA standard, which contain nearly 100% and 0% methylation, respectively, at all CpG sites. The use of these two standards individually or in combination can help identify potential biases in assay development. For established workflows, these standards can serve as a positive and negative control to validate the procedures and results, which is essential in clinical testing to enhance quality control and assurance.

Quality Controls For Validating DNA Methylation Assay Workflows

As a good practice, methylated and non-methylated standards should be processed in parallel to experimental samples as a quality control for the whole workflow. In contrast to standards that are expected to produce a consistent result each time, experimental samples can have many variables, making troubleshooting difficult. In PCR-based and NGS-based DNA methylation assays, inhibitors carried over from the samples, primer design, enzymatic reagents, instrument failures, and many other factors can cause an assay to fail. Data produced by the standards can suggest where to start troubleshooting. For example, if standards perform as expected when run in parallel with experimental samples which perform poorly, the workflow is functioning properly, suggesting the experimental sample quality may be an issue. If standards did not perform as expected, there may be an issue within the workflow that caused the assay to fail.

Controls For Optimizing And Calibrating Bisulfite-Based Assays

Bisulfite PCR (BSP) is a common methodology used for methylation analysis at single-base resolution. Samples are first bisulfite converted and amplified using bisulfite-specific primers. The PCR products generated are sequenced (i.e. Next-Gen Sequencing, Sanger Sequencing, pyrosequencing) and analyzed to determine the methylation status at every cytosine. However, it is critical that primer pairs designed for BSP amplify both methylated and non-methylated sequences with the same efficiency (Figure 1). Otherwise, any amplification bias can result in skewed methylation levels.

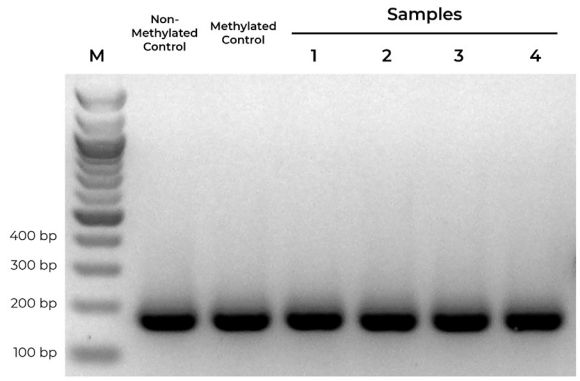

Figure 1. Bisulfite PCR primers should show equally efficient amplification of methylated, non-methylated, or partially methylated templates. Equal amounts of bisulfite converted non-methylated and methylated DNA controls were amplified in parallel with experimental samples using primers designed to target MGMT. Observed bands for methylated and non-methylated templates are of equal intensity, indicating that the primer pair is unbiased.

Using methylated and non-methylated DNA controls, BSP primers can be easily optimized (validated) based on following criteria:

- Specific product amplification
- Equally robust amplification of both methylated and non-methylated DNA standards
- When sequenced, the methylated and non-methylated DNA standards should result in nearly 100% and 0% methylation, respectively. If the results deviate from the expected result, this may indicate PCR bias.

Unlike bisulfite PCR, methylation-specific PCR (MSP) requires methylated and non-methylated templates to be differentially amplified by two different primer sets. One is a methylation-specific primer set, and if amplification occurs, it indicates the amplified region is completely methylated. The second primer set targets non-methylated templates, so any amplification with this primer set indicates the amplified region is completely non-methylated. If amplicons are generated by both primer sets, the region is partially methylated.

Such two primer sets required for MSP can also be optimized and validated utilizing Methylated & Non-Methylated DNA Controls using following guidelines:

- Specific product amplification
- The methylated primer will only amplify the methylated control DNA and will not amplify the non-methylated DNA.
- The non-methylated primer will only amplify the non-methylated control DNA and will not amplify the methylated DNA.

During primer validation, having characterized methylated and non-methylated DNA standards can streamline the process. For example, if a slight PCR signal is observed using the

methylated standard with the non-methylated primers, or vice versa, it would indicate that the primer set is non-specific. For established workflows, especially in clinical assays, methylated and non-methylated DNA standards can serve as positive and negative controls to validate the MSP workflow. Standards will help confirm that the entire process – bisulfite conversion,

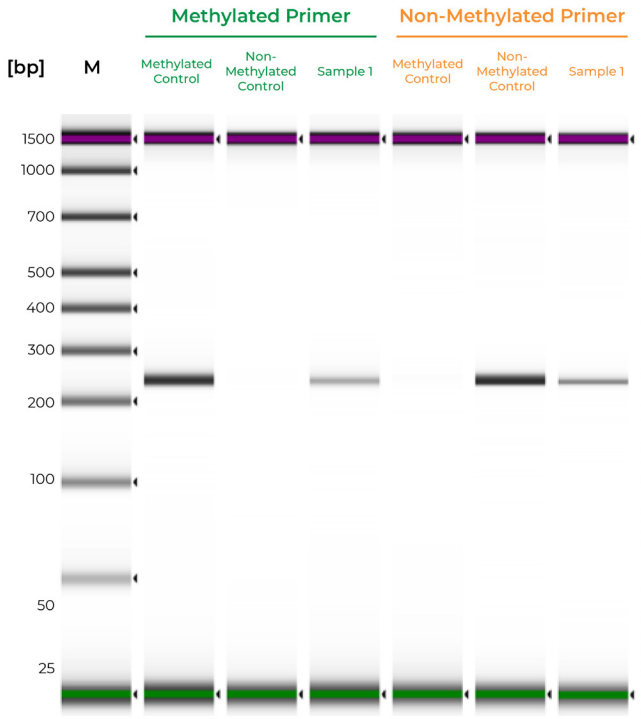

Figure 2. Methylated and non-methylated controls are important for validating the sensitivity of MSP assays. The methylated and non-methylated controls are only amplified by the methylated and non-methylated primers, respectively. Sample 1 is amplified by both primer sets, indicating mixed methylation. Samples were analyzed on the Agilent D100 ScreenTape.

amplification, and analysis – are functioning appropriately, and the results for the test samples are reliable (Figure 2).

In addition to validating primers, DNA standards can be used in place of precious samples to optimize the annealing temperature for new primer sets (Figure 3). An annealing temperature gradient should be performed for each newly designed primer set to ensure optimal amplification of the intended target.

For more tips on primer design, see the Bisulfite Beginner Guide.

Controls For Methylation Sensitive Restriction Enzyme (MSRE) Assays

Methylation sensitive restriction enzyme (MSRE) assays rely on restriction enzymes being able to distinguish methylated versus non-methylated cytosines. If the restriction site is not methylated, the enzyme will digest the DNA while methylated sites will be uncut. Then, methylation at the site can be evaluated using quantitative PCR by comparing the amplification of the digested versus undigested sample. The smaller the ΔC(t) between the two, the higher the methylation levels are, and vice versa (Figure 4). DNA visualization methods,

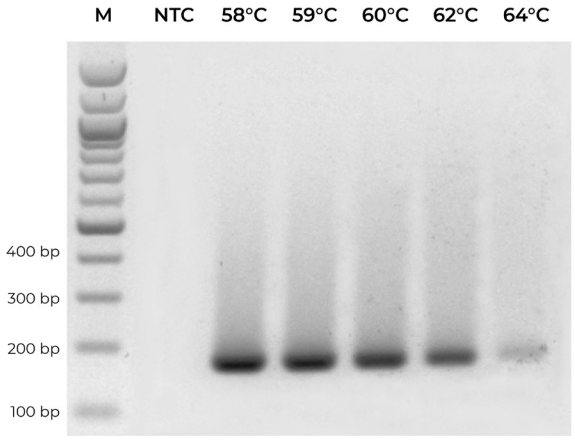

Figure 3. A primer set targeting MGMT was optimized by testing replicate PCR reactions with annealing temperature ranging from 58°C to 64°C.

such as an agarose gel, can also be used to determine if the digestion occurred at the region of interest.

Methylated and non-methylated DNA standards should be digested in parallel to experimental samples to demonstrate the robustness of the restriction enzyme in distinguishing methylated versus non-methylated sites. This will also help determine the sensitivity of the assay to the region of interest.

DNA Standards For Other Applications

DNA standards can be used for optimizing a variety of other applications as it is high-quality genomic DNA with known methylation patterns. This makes them ideal substitutes for precious DNA or problematic sample types when titrating experimental conditions. For example, when optimizing bisulfite library preparation of FFPE DNA, the DNA standards can easily be sonicated or fragmented to be representative of the experimental samples.

The methylated and non-methylated DNA standards have been adapted as controls for numerous methylation assays:

- MethyLight assays[5, 6]
- Methylation-sensitive high-resolution melt analysis[7-9]
- Pyrosequencing[10, 11]
- Methylation arrays[12]
- Methylated DNA Immunoprecipitation[13]
- Bisulfite sequencing library preparation[14]

The Human Methylated & Non-methylated DNA Standards are an example of a validated control set for methylation analysis of human samples. They have been widely used for all the assays mentioned above and much more. Zymo Research also provides a range of other DNA standards that can be used for optimization or quality controls for other assays to help guarantee your data is of the highest quality and ready for publication.

Still unsure of what standards work best for your application? Please email Technical Support (tech@zymoresearch.com) for help!

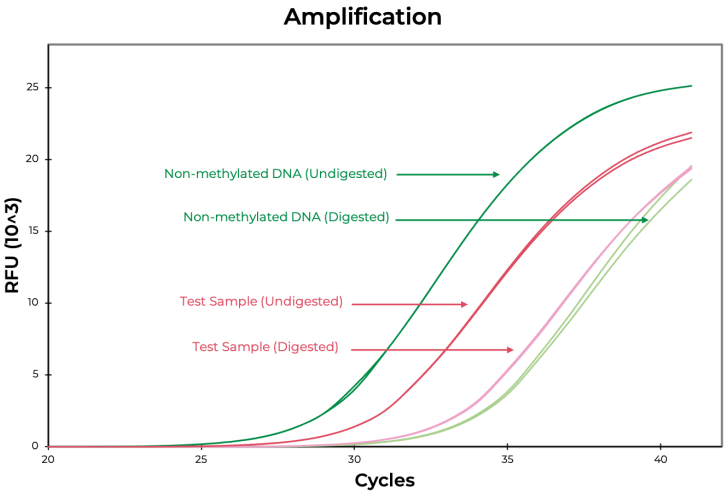

Figure 4. The amplification curve demonstrates the differences in Ct values between digested and undigested samples. The MSRE assay detected 100.9% and 2.7% methylation in the methylated and non-methylated DNA standards, respectively, confirming that the assay worked efficiently. The test sample methylation level was determined to be 17.3.

DNA Methylation Standards Product Guide

Standards	Suggested Applications
Human DNA Standards	
Human Methylated & Non-Methylated DNA Set	• Bisulfite PCR • Methylation-specific PCR • MSRE • Methylation-sensitive HRM analysis
Universal Methylated DNA Standard	• Bisulfite PCR
Human Methylated & Non-Methylated (WGA) DNA Set	• Bisulfite PCR • Methylation-specific PCR
Human Matched DNA Set	• Bisulfite PCR • Methylation assay calibration
Mouse DNA Standards	
Universal Methylated DNA Standard	• Bisulfite PCR
Mouse 5-hmC & 5-mC DNA Set	• Bisulfite PCR • Methylation assay calibration
Escherichia Coli	
E. coli Non-Methylated Genomic DNA	• Monitor bisulfite conversion efficiency (in situ control for NGS bisulfite library) • ELISA standard curves
Artificial DNA Standards	
5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set	• HPLC/Mass Spec • MeDIP
Methylated & Non-methylated pUC19 DNA Set	• Monitor bisulfite conversion efficiency (in situ control for NGS bisulfite library) • MeDIP

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