

# FastStart High Fidelity PCR System Simplifies Study of Epigenetics and DNA Methylation

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## Introduction

Epigenetic mechanisms such as DNA methylation, histone acetylation, and small RNA interference, and their effects on gene activation and inactivation are important in phenotype imprinting, development, and cancer biology [1]. Abnormal methylation of critical genes may lead to severe developmental diseases such as Angelman or Prader-Willi syndrome, and to the development of cancer [2]. The recent interest in clinical aspects of epigenetics has paralleled the emergence of new techniques for studying DNA methylation [3].

Methylated nucleotides can be easily detected by Southern blotting of DNA digested by methylation-specific restriction enzymes. However, not all methylated positions fall within restriction enzyme sequences; moreover, this technique requires substantial amounts of DNA. These problems can be overcome by using methylation-specific PCR (M-PCR) or by sequencing deaminated DNA. In M-PCR, the unmethylated cytosines in a given template DNA are deaminated with bisulfite to uracil, thus changing a CG to a AT pair [4]. Methylated cytosines are resistant to deamination.

To distinguish between methylated and unmethylated genes or promoters, methylation-specific primers for different sizes of amplicons are used. The amplification of a single gene copy by PCR offers high sensitivity, and – with stringent template handling and primer design – high specificity. The main obstacles to a wider use of M-PCR are difficulties in DNA deamination, and purification of deaminated DNA, as well as laborious and expensive optimization [5].

We have devoted a substantial amount of time, resources, and energy to apply M-PCR in the study of male infertility genetics in our laboratory; therefore, we would like to share our positive experience with the FastStart High Fidelity PCR System, which seems to be an optimal solution for M-PCR.

We tested the hypothesis that the methylation pattern (inactivation) of the X chromosome-inactivating transcript

(XIST) located on the X chromosome in females (46, XX) and males with Klinefelter syndrome (KS) (47, XXY) is the same [6].

## Materials and Methods

DNA from peripheral blood lymphocytes of healthy males and females, and from men with KS was extracted using commercially available kits. The DNA was stored at  $-20^{\circ}\text{C}$ .

### DNA deamination

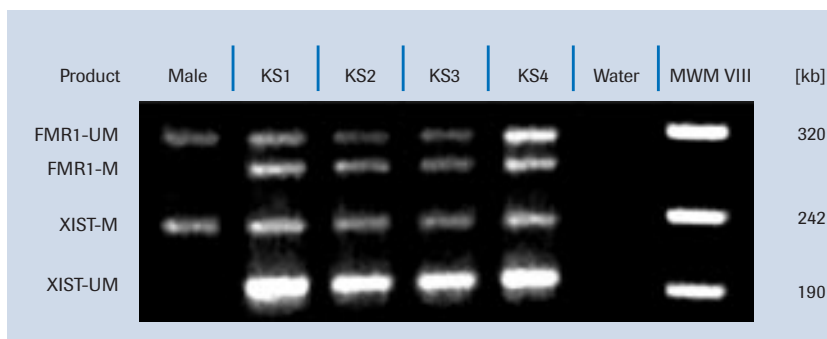
DNA (0.5–1 mg in 50  $\mu\text{l}$   $\text{H}_2\text{O}$ ) was denatured at  $37^{\circ}\text{C}$  (17 minutes) and  $55^{\circ}\text{C}$  (2 minutes) with 5.5  $\mu\text{l}$  of 2M NaOH. Subsequently, 30  $\mu\text{l}$  of 10 mM hydroquinone and 520  $\mu\text{l}$  of 3 M sodium hydrogensulfite, both freshly prepared, were added to the DNA solution and overlaid with mineral oil. The mixture was incubated in a water bath at  $55^{\circ}\text{C}$  overnight (in darkness). The reaction was terminated by adding NaOH during the purification process. Deaminated DNA was suspended in Tris-EDTA and stored at  $-20^{\circ}\text{C}$ .

### Methylation-specific PCR

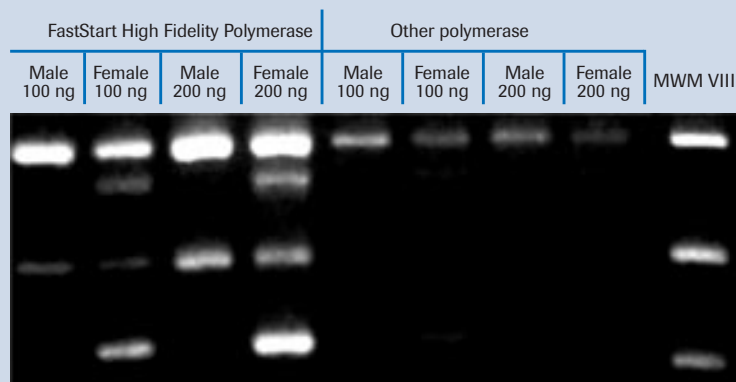
We used methylation-specific primers for the gene of interest (XIST) and for a control gene with an opposite pattern of methylation (familial mental retardation gene 1, FMR1) [7].



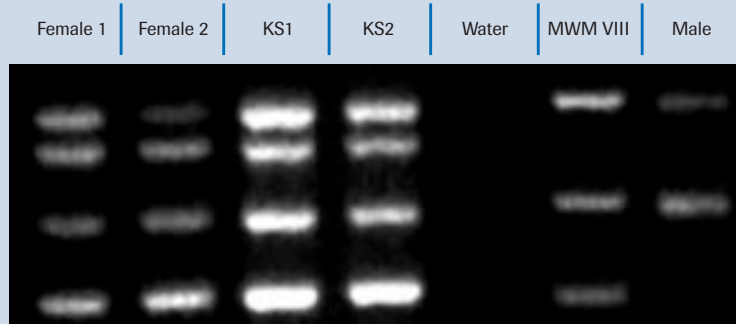
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**Figure 1: Consistency.** 10  $\mu\text{l}$  of DNA were mixed with 2  $\mu\text{l}$  of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. The FastStart High Fidelity PCR Kit yields consistent results in M-PCR. A healthy male has only one X chromosome, thus M-PCR shows only one band for methylated XIST (XIST-M). The FMR1 gene has an opposite methylation pattern and serves as a control (KS1, KS2, KS3, KS4 – template DNA from subjects with Klinefelter syndrome).



**Figure 2: Comparison with other polymerases.** 10  $\mu$ l of DNA were mixed with 2  $\mu$ l of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. FastStart High Fidelity Polymerase yields superior results in M-PCR. FastStart High Fidelity Polymerase was compared with competitor products using the same template DNA. No optimization steps are necessary.



**Figure 3: Methylation pattern.** 10  $\mu$ l of DNA were mixed with 2  $\mu$ l of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. The restriction analysis using FastStart High Fidelity Polymerase shows that men with KS (KS1, KS2) have the same pattern of XIST methylation as females (female 1, female 2).

The PCR master mix was prepared with 2.5  $\mu$ l 10x buffer mix (final Mg concentration: 1.8 mM), 0.5  $\mu$ l dNTPs, and 0.25  $\mu$ l FastStart HighFidelity Polymerase, and water to final volume of 25  $\mu$ l for each reaction. 200 ng DNA template in 4  $\mu$ l water was mixed with 2  $\mu$ l primer multiplex mix. The PCR master mix was added to the DNA and primer mix. PCR was performed in a thermal cycler using the following settings: 95°C, 1 x 3 minutes, (95°C/30 seconds, 61°C/40 seconds, 72°C/50 seconds) x 40, 1 x 71°C/7 minutes, 4°C for FastStart High Fidelity Polymerase. The PCR master mixes for other polymerases tested were prepared according to manufactures' instructions.

Some authors suggested using specialized M-PCR reaction buffer with 0.1% Triton-X and ammonium sulfate (5-30 mM); however, in our experiment, the addition of Triton-X or ammonium sulfate had a negative impact on the yield across all tested polymerases.

## Results

Methylation-specific PCR was 100% specific and 100% sensitive in detecting X chromosome polysomy in males with nonmosaic KS. The DNA from males showed two bands on an agarose gel: one for the unmethylated FMR1 promoter (FMR1-UM) and one for the methylated XIST gene (XIST-M) (Figure 1). The DNA from females and males with KS showed four bands: methylated and unmethylated FMR1 promoter (FMR1-UM, FMR1-M), and methylated and unmethylated XIST gene (XIST-M, XIST-UM, Figure 1-3). The pattern of XIST methylation in men with KS was the same as that in females (Figure 3).

The FastStart High Fidelity PCR System yields the best results in methylation-specific PCR as compared with other available polymerases (Figure 2).

## Conclusions

The FastStart High Fidelity PCR System is a highly reliable and cost efficient option for research application using methylation-specific PCR. The FastStart High Fidelity PCR System has worked "out of box" with minimal optimization. Adequate deamination and careful primer design are necessary. In multiplex M-PCR, the primer concentrations have to be established empirically, especially for semi-quantitative analysis of methylation patterns. In our laboratory, we have expanded the application to other genes involved in male infertility achieving an excellent reproducibility of results. We strongly believe that this report should lead to the development of new applications using FastStart High Fidelity PCR System-based M-PCR in cancer research, developmental biology, and other areas of the life sciences. ■

## References

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Product	Pack Size	Cat. No.
<b>FastStart High Fidelity PCR System</b>	1 kit (50 reactions)	03 553 426 001
	1 kit (200 reactions)	03 553 400 001
	1 kit (1000 reactions)	03 553 361 001
	1 kit (1000 reactions)	03 553 361 001

