KimanTech Technical Application:
1-Step vs 2-Step PCR for DNA Methylation Detection

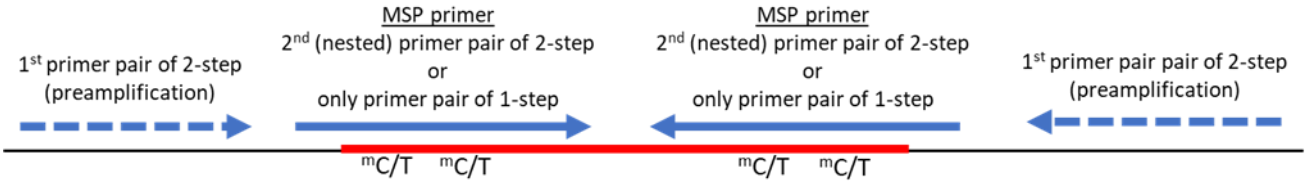
Background

DNA methylation is a form of epigenetics that is widely studied in development and cancer research. The most common type of DNA methylation is a methyl group added to the 5 position of the C base in CpG dinucleotides. CpG nucleotides are often clustered in transcriptional regulatory regions of genes. Variability in CpG methylation patterns is a major component in gene expression regulation. The most common methods used to determine the DNA methylation pattern involve treating a DNA sample with bisulfite, which converts all non-methylated C bases to U(T) residues, while leaving methylated C bases intact. Differentiation of C or T bases can subsequently be determined by biochemical methods such as PCR, sequencing, or DNA melting analysis. A common PCR method is Methylation Specific PCR (MSP) in which the primer binding sites encompass the CpG residues of interest. Multiple primers are designed that correspond to different forms of methylation such that the methylation status can be determined by which primer pairs successfully amplify the sample of interest.

Draht et al. (Clinical Epigenetics 8:44, 2016) report that two steps of PCR utilizing a preamplification with non-MSP primers followed by MSP can be more robust than a single MSP step, particularly when the samples derive from FFPE tissue samples. In this study we compared the sensitivity of single-step MSP vs. two-step MSP. PCR methods utilizing bisulfite converted DNA are particularly relevant to the Alluvia system since the bisulfite treatment incorporates uracil into the templates and therefore the Uracil-DNA-Glycosylase PCR anti-contamination system cannot be utilized.

Experimental Design and Methods:

Fig. 1. The PCR strategy used in this study:



- MSP primer sequences from 5 gene loci (CHAD, GFI1, MX2, NEU1 & VWCE) were utilized, each had been designed for nested MSP (in a single-plex format for both steps).
- Two types of DNA templates were utilized:
- Purified human DNA - fully methylated or fully unmethylated (from ZymoResearch);
- Purified human DNA from FFPE tissue.
- DNA from FFPE samples was purified using the Roche High Pure FFPET DNA isolation kit, then bisulfite treated and column purified using the Zymo EZ DNA Methylation-Lightning kit.
- ABI Power SYBR GreenTM mixture used for all PCR amplifications
- Two-step (nested) MSP:

  Step 1: 5-plex non-MSP using outer primers (40 cycles, anneal at 56C)

  Step 2: Dilute PCR 100X in TE buffer

  Step 3: Monoplex MSP with inner primers (20-40 cycles, anneal at 66C)

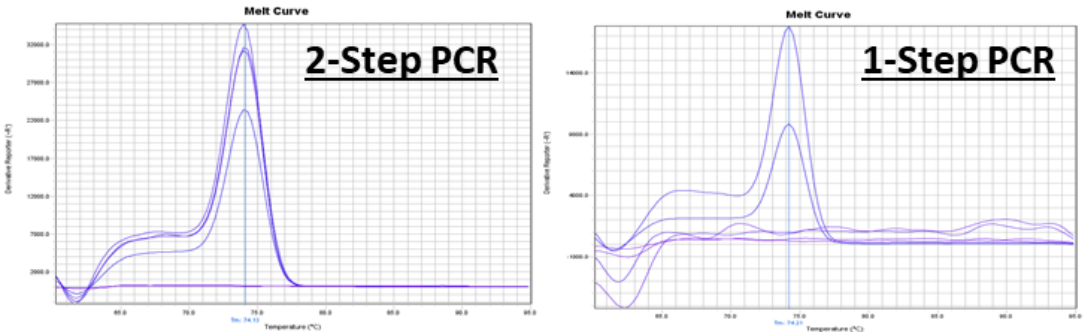
- Single-step (direct) MSP:

Step 1: Monoplex MSP) with inner primers (20-40 cycles, anneal at 66C)

Results:

Fig. 2: Representative Results - Limit Of Detection studies: qPCR (**A.**) and gel electrophoresis data; (**B.**) A positive result had clear peaks at the expected melting temperature and the expected size gel bands.

A.



B.

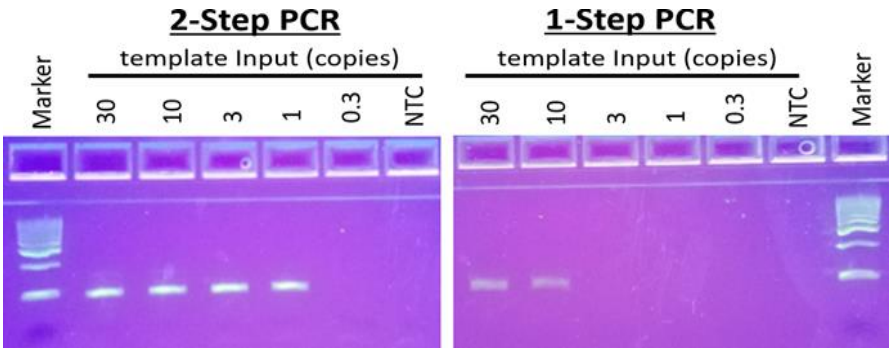


Fig. 3: Limit Of Detection studies: For each cell in the above tables, a DNA templated dilution series was performed. The dilution with the least amount of template is indicated.

Limit Of Detection by Methylation Specific PCR (Numbers indicate copies of a fully methylated or fully unmethylated DNA template - from Zymo Research. UD = UnDetected)								
	Methylation Specific Primer Set				**Non-methylation Specific Primer Set**			
	Methylated DNA		Unmethylated DNA		Methylated DNA		**Unmethylated DNA**	
Target Gene	**2-Step PCR**	**1-Step PCR**	**2-Step PCR**	**1-Step PCR**	**2-Step PCR**	**1-Step PCR**	**2-Step PCR**	**1-Step PCR**
CHAD	10	300	UD	UD	UD	UD	10	300
GFI1	1	300	UD	UD	UD	UD	10	300
MX2	3	30	UD	UD	UD	UD	1	300
NEU1	1	10	UD	UD	UD	UD	10	300
VWCE	1	30	UD	UD	UD	UD	10	300

Limit Of Detection by Methylation Specific PCR (Numbers indicate copies of a FFPET derived bisulfite treated DNA template - concentration by Pico Green. UD = UnDetected)								
	Methylation Specific Primer Set				Non-methylation Specific Primer Set			
	Normal FFPE Tissue		Tumor FFPE Tissue		Normal FFPE Tissue		Tumor FFPE Tissue	
Target Gene	2-Step PCR	1-Step PCR	2-Step PCR	1-Step PCR	2-Step PCR	1-Step PCR	2-Step PCR	1-Step PCR
CHAD	1000	UD	UD	UD	UD	UD	UD	UD
GFI1	UD	UD	UD	UD	UD	UD	UD	UD
MX2	30	UD	3000	UD	100	UD	1000	UD
NEU1	UD	UD	UD	UD	UD	UD	UD	UD
VWCE	UD	UD	UD	UD	100	UD	UD	UD

Fig. 4: Template ratio tests: The indicated mixtures of fully methylated and unmethylated (ZymoReasearch) DNA templates were tested using the GSTP1 methylation specific primer pairs and electrophoresed on a gel.