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

Background:

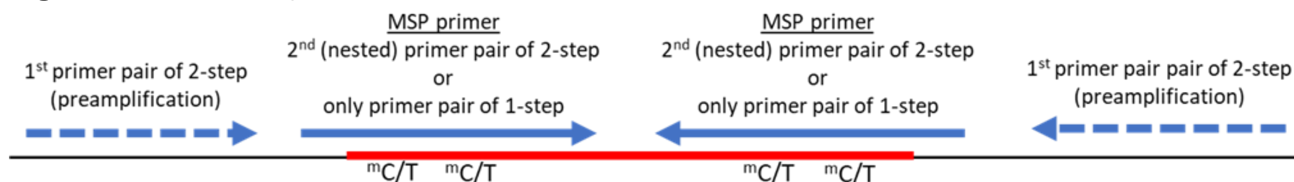
DNA methylation is a form of epigenetics that is widely studied in cancer research and developmental biology. The predominant form of DNA methylation is a methyl group added to the 5 position of the Cytosine base in CpG dinucleotides, which are often clustered in transcriptional regulatory regions. Variability in CpG methylation patterns is a major component in gene expression regulation. The common methods to determine the DNA methylation patterns involve treating a DNA sample with bisulfite, which converts all non-methylated C bases to U(T) residues while leaving methylated C bases intact, and subsequent differentiation of C or T bases by PCR, sequencing, or DNA melting analysis.

Methylation Specific PCR (MSP) involves designing two primers, one specific for the methylated template (a C base) and one specific for the unmethylated template (a T base). The methylation status can be determined by which primer successfully amplifies the sample of interest. Previous reports have shown that utilizing two steps of PCR including a preamplification with outer non-MSP primers followed by a second PCR step using nested MSP primers can be more robust than a single MSP step (Palmisano *et al.*, Cancer Res. 60:5954, 2000), particularly when the samples derive from FFPE tissue samples (Draht *et al.*, Clinical Epigenetics 8:44, 2016). In this study we compared the sensitivity and specificity of 1-step MSP vs. 2-step MSP.

Two-step PCR methods that use bisulfite treated DNA derived from FFPE tissue are particularly prone to PCR product contamination because both the formalin and bisulfite treatments degrade the sample DNA reducing PCR efficiency relative to a potential PCR product contaminant. To make matters worse, the Uracil-DNA-Glycosylase anti-PCR product contamination system cannot be utilized because the bisulfite treatment incorporates uracil into all templates. Therefore, the continuous PCR product containment provided by the Alluvia system is particularly attractive for MSP applications.

Experimental Design and Methods:

Fig. 1. The PCR strategy:



- Previously described MSP primer sequences from 5 gene loci (CHAD, GFI1, MX2, NEU1 & VWCE) were utilized. These PCR amplicons had been designed for nested MSP (Ruijter *et al.*, Lab Invest 95:833, 2015).
- Two types of human DNA templates were utilized:
  - Fully methylated and fully unmethylated (purchased from ZymoResearch),
  - Purified from FFPE tissue (using the Roche High Pure FFPET DNA isolation kit).
- DNA was bisulfite treated and column purified (Zymo EZ DNA Methylation-Lightning kit).
- Two-step MSP:
  - Step 1: Five-plex non-MSP using outer primers
  - Step 2: Dilute PCR 100X, monoplex MSP with nested primers

- Single-step MSP:

  Step 1: Monoplex MSP with inner (nested) primers

- ABI Power SYBR Green™ mixture was used for all PCR amplifications

Results:

Fig. 2: Representative Results - Limit Of Detection (LOD) studies: (A.) qPCR and (B.) gel electrophoresis. To be considered a positive result, a given dilution had a clear peak at the expected melting temperature and a gel band at the expected size.

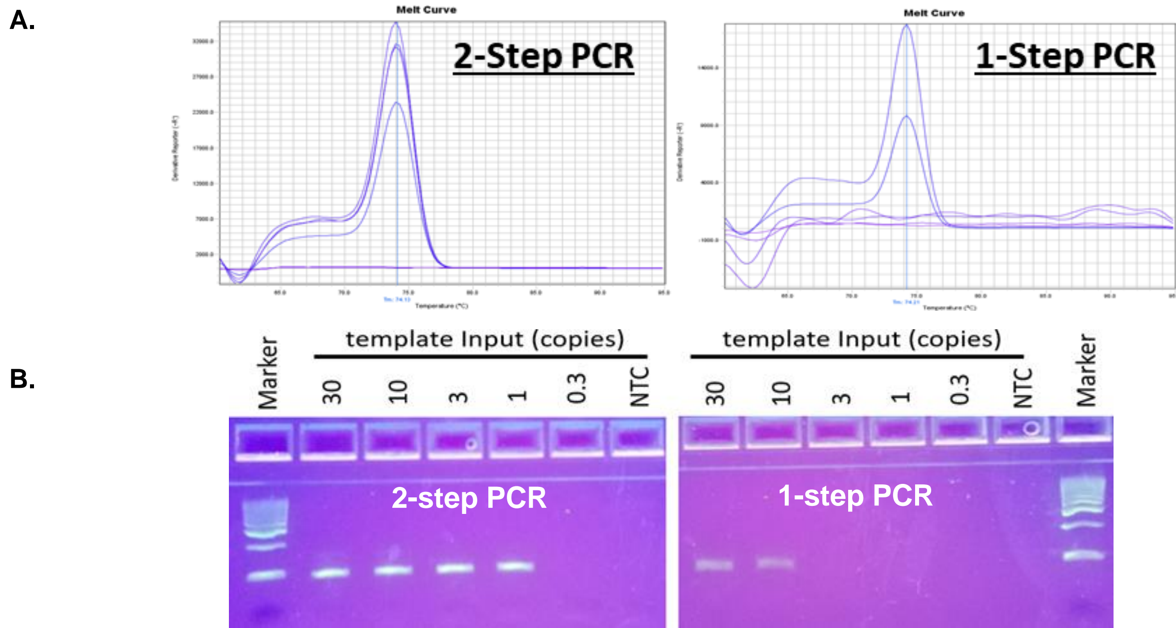
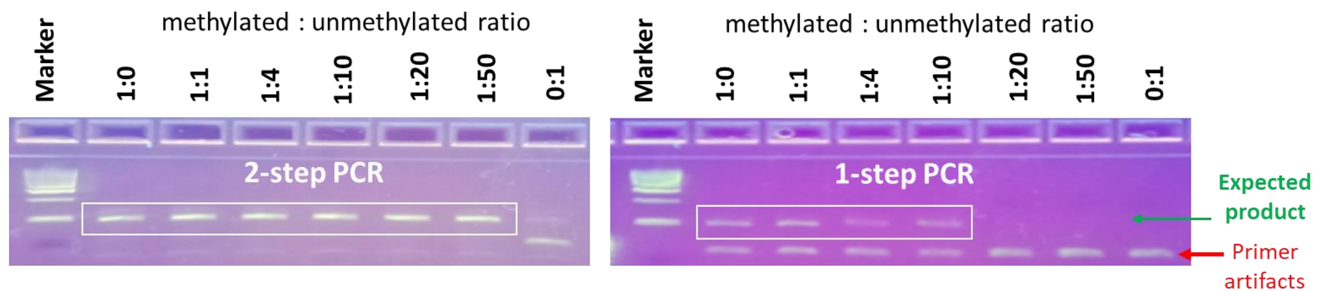


Fig. 3: Limit Of Detection- template copies: For each cell in the tables below, a DNA templated dilution series was performed. The dilution with the least amount of template is indicated. Numbers indicated DNA template copies in the first PCR step.

LOD by MSP - copies of fully methylated or fully unmethylated DNA (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

LOD by MSP - copies of a FFPET derived DNA template (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: Limit Of Detection - template ratios: The indicated mixtures of fully methylated and unmethylated (ZymoReasearch) DNA templates were amplified using the GSTP1 fully methylated specific PCR amplicon and electrophoresed on a gel.



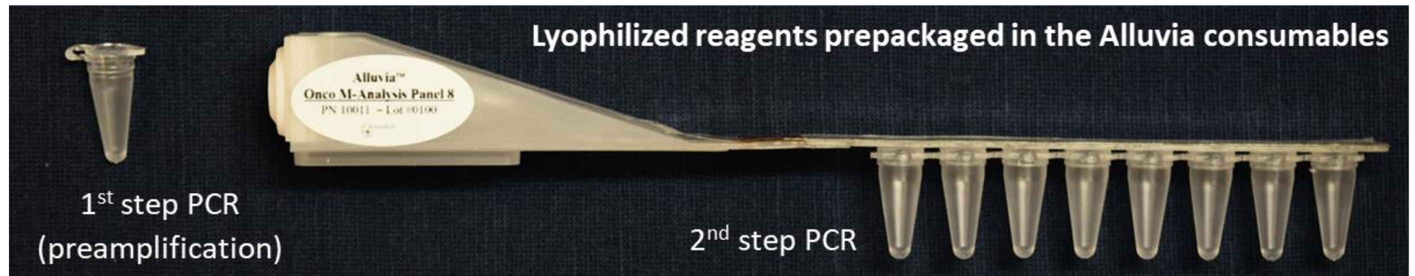
Discussion:

Results Summary

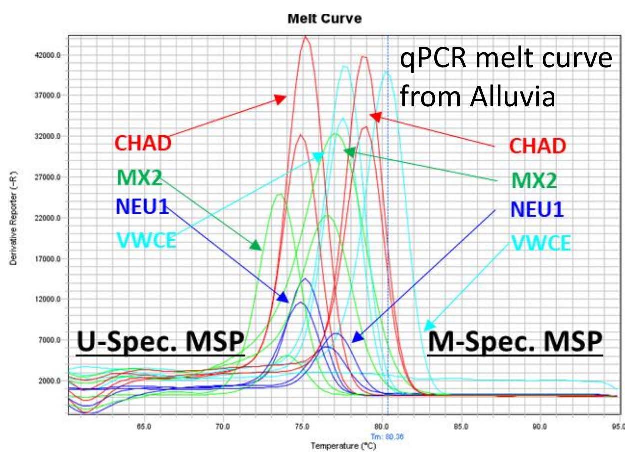
- The MSP primers were very specific for the appropriate form of methylation.
- Sensitivity of 2-step PCR ranged from 1 to 10 DNA template copies (added to the first PCR step), whereas sensitivity of 1-step PCR ranged from 10 to 300 template copies. Average sensitivity increase of 2-step MSP was 30-fold.
- Sensitivity dropped significantly when using FFPET derived DNA. However, sensitivity using 2-step MSP (as few as 30 template copies) was still significantly better than 1-step MSP (unable to detect a signal from any sample concentration).
- Specificity of 2-step MSP (using ratios of methylated and unmethylated templates) also appeared significantly improved compared to 1-step MSP as 2-step MSP could detect 1 copy of of 50 whereas 1-step MSP could detect only 1 copy of 10.
- Our results are consistent with previous reports (Palmisano *et al.*, Draht *et al.*) that 2-step MSP PCR has significant sensitivity advantages over 1-step MSP.

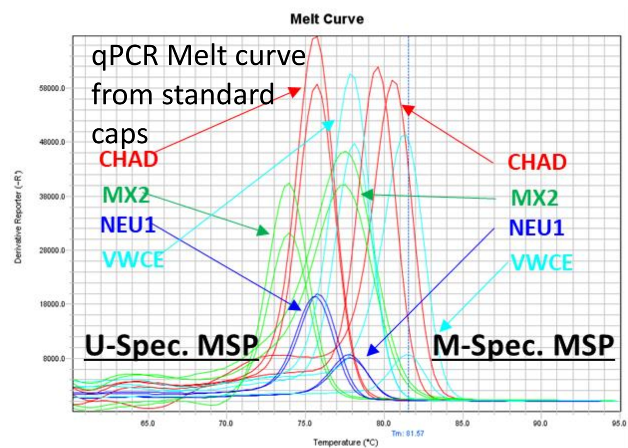
The MSP Assay in a Prepackaged Format

A prepackaged MSP assay using the amplicons for the genes VWCE, MX2, NEU1, and CHAD was developed by lyophilizing the primers, dNTPs, buffer, and Taq polymerase in both a single PCR tube (for the multiplex preamplification) and an 8-well PCR tube strip (for the 2nd-step nested MSP). The performance of this assay was compared using an Alluvia consumable versus a standard cap strip.



Following qMSP, a subsequent melting analysis demonstrate that the Alluvia System produces similar results as standard PCR tube strip caps.





Compared to a standard cap strip, the Alluvia system has the advantages of:

- Continuous containment:
  - Eliminate PCR product contamination,
  - The prepackaged biochemistry remains sealed throughout usage.
- Reduced pipetting steps and associated errors.
- An attractive intuitive form factor.