Sep 07, 2020

Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP

Hector Hernandez-Vargas[1], Chloe Goldsmith[2]

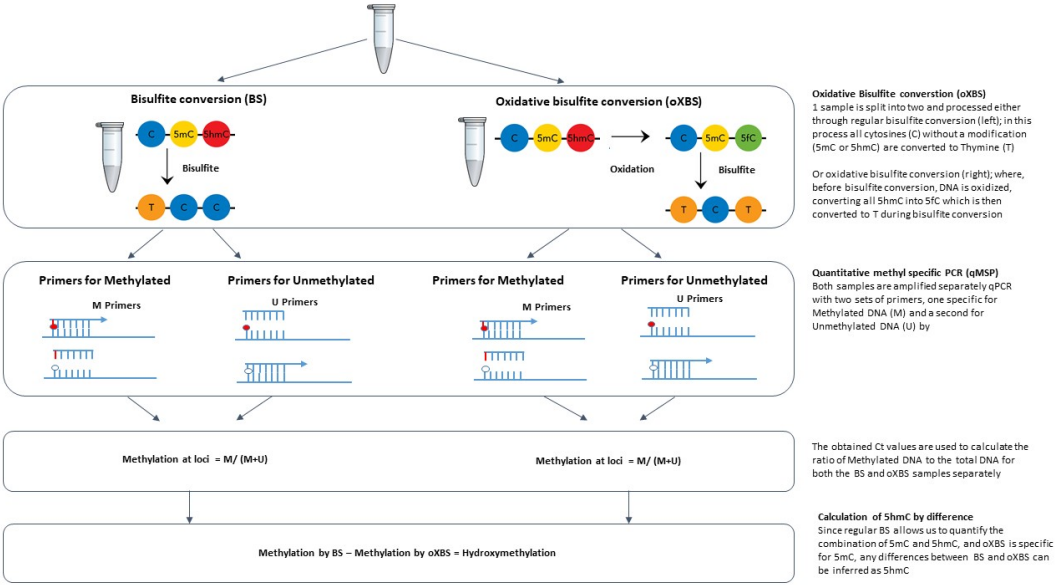
[1]Centre Léon Bérard (CLB); [2]Cancer Research Center of Lyon (CRCL) FRANCE

2 *Works for me* dx.doi.org/10.17504/protocols.io.52bg8an

Chloe Goldsmith

ABSTRACT

There are several tools for investigating the methylation status of genomic DNA; many of these are expensive, do not allow for quantification or are unable to distinguish intermediary methylation metabolites such as 5-hydroxymethylation. Since significant changes in methylation status can be very small, the development of highly sensitive techniques is important for validating high throughput base resolution techniques. To this end, we developed a quantitative methyl specific PCR method based on oxidative bisulfite converted DNA. By designing primers specific for methylated and unmethylated DNA we are able to quantify the "real" methylation (5mC) and the hydroxymethylation (5hmC) at specific loci. This technique is important for understanding the methylation status of a region quickly and also as a tool for validating methyl-seq data.



DOI

dx.doi.org/10.17504/protocols.io.52bg8an

PROTOCOL CITATION

Hector Hernandez-Vargas, Chloe Goldsmith 2020. Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. **protocols.io** https://dx.doi.org/10.17504/protocols.io.52bg8an

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

KEYWORDS

5mC, 5hmC, oxidative bisulfite, quantitative methyl specific PCR, epigenetics, detecting DNA base modifications

CREATED

Jul 31, 2019

LAST MODIFIED

Sep 07, 2020

PROTOCOL INTEGER ID

26403

GUIDELINES

This protocol explains through the necessary steps involved in quantifying methylation and hyroxymethylation at specific loci, such as in the promoter of a gene of interest; optimised to be cheap and quick while only requiring minimal sample DNA contents. Thus, this protocol is useful for valdating methyl-seq data when samples are valuable and only low starting materials can be obtained.

Introduction

DNA methylation (5mC) is a well described epigenetic mark with important functions including its involvement in gene silencing. However, there are also other DNA base modifications where less is known about their function and activity; such as DNA hydroxymethylation (5hmC). 5hmC is formed by the oxidation of 5mC by Ten-Eleven Translocase (TET) proteins. Evidence suggests that 5hmC is an intermediate of DNA de-methylation and is also involved in stem cell pluripotency.

In recent years, there have been several techniques developed to study 5mC and 5hmC; most of which, involve enzymatic digestion and chemical pull-down of fragmented DNA. Moreover, these techniques often require large amounts of starting material, which, in clinical settings is often not possible. Bisulfite conversion involves treating DNA with sodium bisulfite converting all unmethylated cytosies to thymine, while leaving methylated cytosines unchanged. This technique is usefull for smaller quantities of DNA, and hence is widely used to quantify 5mC, however, it is not able to distinguish the difference between 5mC and 5hmC. This lead to the development of oxidative bisulfite conversion. Whereby, the DNA is first oxidized, converting all 5hmC to 5fC which can then also be converted to thymine in the same way as regular cytosines during sodium bisulfite treatment. Therefore, if a parallel reaction for regular bisulfite is conducted, the amount of 5hmC can be calculated by difference.

Hence here, we use the oxidative bislufite technique which is now a well described tool for conversion of DNA prior to several techniques for determining whole genome levels of 5mC and 5hmC. To add to this valuable tool, we have developed a quantitative methyl specific PCR method (qMSP) to efficiently quantify the amount of these epigenetic marks at specific loci. Whereby, the converted DNA is amplified by PCR with two sets of primers, one pair designed for methylated DNA and a second designed for unmethylated DNA.

This technique is not only fast and inexpensive, but, can also be useful for low inputs. However, some optimization is required before handling the more precious samples in order to ensure reproducibility of the developed assays. Therefore our aim here, is to outline the necessary steps for optimization of a qMSP assay.

MATERIALS

NAME	CATALOG #	VENDOR
REPLI-g Cell WGA & WTA	150052	Qiagen
Agencourt Ampure XP	A63AA0	Beckman Coulter
Acetonitrile HPLC	9012-03	fisher
TrueMethyl oXBS conversion kit	0414-32	Tecan

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

NAME	CATALOG #	VENDOR
iTaq Universal SYBR Green Supermix	172-5112	BioRad Sciences
5mC 5hmC and non-methylated synthetic control DNA	D5405	Zymo Research
S-adenosylmethionine (SAM)	E2010)	Zymo Research
Primers for synthetic DNA controls (list in Table 1) and target loci		Invitrogen - Thermo Fisher

MATERIALS TEXT

The following table is the list of qMSP primers designed by our team manually. These are needed for the synthetic control DNA.

Primer name	Direction	Sequence
Methylated Primer (and no BS)	F	GATCGGGAAGGGCTGGATTTGCT
Methylated Primer (and no BS)	R	CCATTTGCCCCATATCACCGTCG
Unmethylated Primer (and 5hmC)	F	GATTGGGAAGGGTTGGATTTGTT
Unmethylated Primer (and 5hmC)	R	ACCATTTACCCCATATCACCATC

Table 1. Primer sequences for synthetic control DNA

This DNA is unique in that every cytosine is either methylated, unmethylated or hydroxymethylated. Hence, the primers for methylated DNA after bisulfite conversion are the same as the primers needed for non-modified DNA (since methylated cytosines are resistant to bisulfite conversion and unmethylated cytosines are converted to T's). Moreover, hydroxymethylation is recognized with the same primers used for unmethylated DNA, since after oxidative bisulfite conversion, all hydroxymethylated cytosines will be converted to T's, the same as non-methylated cytosines.

In addition to these primers, unique primers must be designed for your region of interest. How to do this is outlined in detail in step 1.

BEFORE STARTING

Before you start, the desired regions of interest for methylation (5mC) and hydroxymethylation (5hmC) quantification must be identified and primers designed to amplify methylated and unmethylated DNA. STEP 1 in this protocol details the steps needed to undertake this exercise.

Primer Design for region of interrest

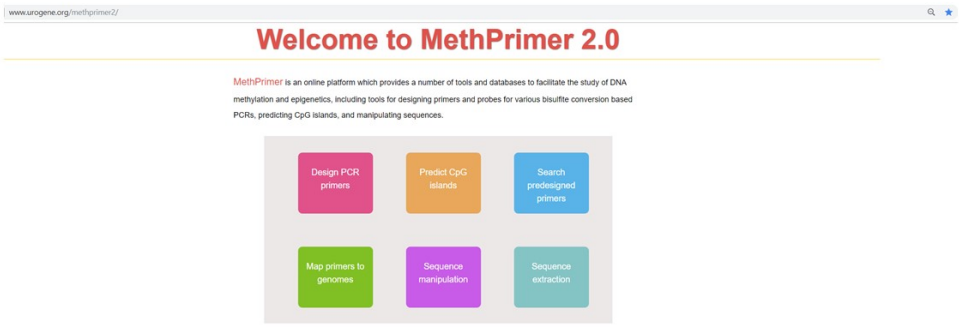
1 ***Primer design***

FASTA records for the target regions should be identified and can be taken from UCSC genome browser. Primers for methylated and unmethylated DNA (MSP primers) can be designed using available online tools including methprimer.

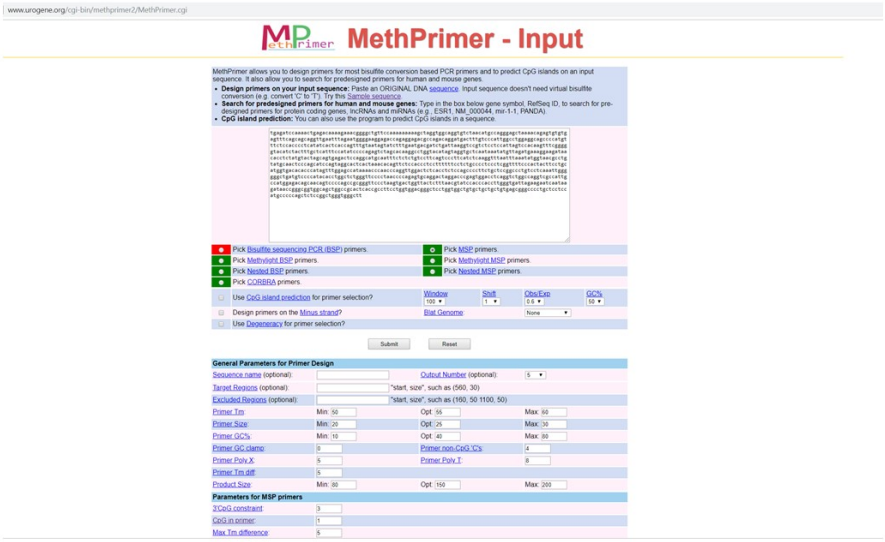
NOTE: Primer design has a huge impact on the specificity of the reaction. It is important to know your target region well and follow the rules for primer design closely to ensure primer stability and the avoidance of secondary structures.
It is also possible to design primers and probes for taqman assays to further increase specificity, however, the assay we are outlining here is designed as a fast and inexpensive tool. Therefore, we have optimized based on SYBR green and basic primers with sufficient efficacy (as indicated by our control experiments (figure 1).

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

1.1 Once the sequence for target region of interest is obtained, go to the methprimer website to design primers for MSP and select "Design PCR primers" (Pink)



1.2 Paste the FASTA record into the available space and select "Pick MSP primers"

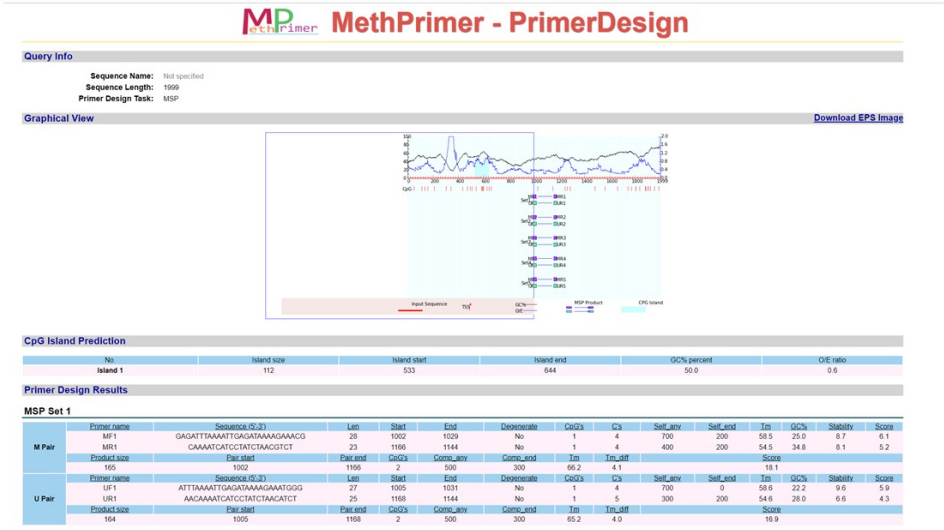


Here you can enter specifics for the primers including desired melting temps, product size ect. Default settings for MSP should be sufficient for most cases. An essential consideration for MSP primers is they must include at least 1 CpG site in the sequence in order to detect if there is methylation or not. More information can be found in the help section for MethPrimer.

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

1.3 Selecting the Primer sequences

Meth Primer will suggest primer sequences for both methylated and unmethylated DNA. It is important to order both the methylated and unmethylated primers since two PCRs will be performed per sample. One for the methylated and another for the unmethylated.



MethPrimer - PrimerDesign

Query Info

Sequence Name: Not specified
Sequence Length: 1999
Primer Design Task: MSP

Graphical View — Download EPS Image

CpG Island Prediction

No	Island size	Island start	Island end	GC% percent	O/E ratio
Island 1	112	533	644	50.0	0.6

Primer Design Results

MSP Set 1

	Primer name	Sequence (5'-3')	Len	Start	End	Degenerate	CpG's	C's	Self_any	Self_end	Tm	GC%	Stability	Score
M Pair	MF1	GAGATTTAAAATTGAGATAAAAGAAACG	28	1002	1029	No	1	4	700	200	58.5	25.0	8.7	6.1
	MR1	CAAAATCATCCTATCTAACGTCT	23	1166	1144	No	1	4	400	200	54.5	34.8	8.1	5.2
	Product size	Pair start	Pair end	CpG's	Comp_any	Comp_end	Tm	Tm_diff			Score			
	165	1002	1166	2	500	300	66.2	4.1			18.1			
U Pair	Primer name	Sequence (5'-3')	Len	Start	End	Degenerate	CpG's	C's	Self_any	Self_end	Tm	GC%	Stability	Score
	UF1	ATTTAAAATTGAGATAAAAGAAATGGG	27	1005	1031	No	1	4	700	0	58.6	22.2	9.6	5.9
	UR1	AACAAAATCATCCTATCTAACATCT	25	1168	1144	No	1	5	300	200	54.6	28.0	6.6	4.3
	Product size	Pair start	Pair end	CpG's	Comp_any	Comp_end	Tm	Tm_diff			Score			
	164	1005	1168	2	500	300	65.2	4.0			16.9			

Once you have selected the ideal primer sequences for your experiment, the oligos can be ordered through your preferred platform such as Invitrogen and optimised for melting temp and concentration as with regular primers for qPCR.

Preparing Control DNA

2 ***Fully unmethylated DNA control***

Several controls are necessary to ensure your experiments are working efficiently.

Whole genome amplification is sufficient to synthesize unmethylated DNA controls. We recommend the Qiagen REPLI-g whole genome amplification kit. PCR amplification should be performed according to manufacturer's instructions.

2.1 The following link is to the detailed protocol for WGA needed to prepare a fully unmethylated sample. Starting material 1-10ng of DNA. The steps are outlined in the sub-sections to follow, however, reading the original protocol before-hand is advised.

https://www.qiagen.com/fr/service-and-support/learning-hub/technologies-and-research-topics/wga/replig-principal-procedure/#REPLIg-protocols

Prepare sufficient Buffer D1 and N1 for the total number of reactions seperately

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

2.2

Component	Buffer D1	Buffer N1
Reconstituted Buffer DLB	9 µL	-
Stop solution	-	12 µL
Nuclease-Free water	32 µL	68 µL
Total Volume	41 µL	80 µL

2.3 Place **5 µl** of template into microcentrifuge tube with **5 µl** of Buffer D1 (prepared in step 2.2)

2.4 Incubate at room temperature for **00:03:00**

2.5 Add **10 µl** of the buffer N1 (prepared in step 2.2) and mix by vortexing

2.6 Prepare master mix (MM) on ice by adding

1 µl of REPLI-g Mini DNA Polymerase to

29 µl of REPLI-g Mini reaction Buffer

2.7 Add MM from step 2.6 **30 µl** to denatured DNA **20 µl**

Total volume now **50 µl**

2.8 Incubate at **30 °C** for **16:00:00**

And then inactivate polymerase by heating to **65 °C** for **00:03:00**

Amplified DNA can be stored at **-20 °C** until further analysis.

3 *Fully methylated DNA control*

DNA can be methylated to make a fully methylated control by using the enzyme SAM (S-adenosylmethionine) (ZYMO cat# E2010). Ideally the unmethylated DNA synthesized in the last step should be used for this reaction.

3.1 Standard Reaction Setup: The setup below outlines a typical CpG methylase reaction in a 20 µl final reaction volume. The reaction volumes can be adjusted accordingly depending on experimental requirements.

Prepare reagents according to the following:

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

Reagent:	**µL**
10X CpG Reaction Buffer	2
20X SAM (S-adenosylmethionine), 12 mM	1
DNA at 100-250 ng/µl (1000ng maximum)	4
CpG Methylase (4 units/µl)	1
Water	12

3.2 Incubate at **30 °C for 4 hours**

3.3 Add an additional **1 µl** 20X SAM (S-adenosylmethionine), 12 mM

3.4 Incubate at **30 °C for 4 hours**

Fully methylated DNA can be stored at **-20 °C** until further analysis.

4 ***Fully methylated, fully hydroxymethylated and fully unmethylated stanandards (synthetic DNA controls)***

In addition to the fully methylated and unmethylated controls, it is also important to have a control for the oXBS conversion. For this, it is possible to use synthetic DNA controls in order to efficiently quantify 5hmC. This DNA is unique since each cytosine in the respective samples is either methylated, unmethylated or hydroxymethylated, and not just in the context of CpG's.
The primers for methylated and unmethylated were designed manually and sequences are provided in the table in the methods section, which, should be ordered along with the sequences designed for target regions.

NOTE: These synthetic DNA standards come fully methylated, unmethylated or hydroxymethylated, however, they still need to be BS and oXBS converted (step 5) the same as all DNA samples as well as the fully methylated and unmethylated controls.

oXBS conversion

5 ***Oxidative bisulfite conversion of DNA samples***

The following link is to the Nugen oXBS protocol for conversion of DNA:

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

https://www.nugen.com/sites/default/files/M01481_v3_User_Guide%3A_TrueMethyl_oxBS_module_5911.pdf

In this protocol, each sample of DNA is split into two so that, in parallel, two reactions are conducted; regular bisulfite conversion (BS) as well as oxidative bisulfite conversion (oXBS). For more information see the guidelines and graphical abstract in this protocol and follow the link above.

100 ng - **1000 ng** of each **sample, control or standard** should be carried through the Nugen oXBS protocol according to manufacturers instructions.

For controls and standards, we recommend taking **1000 ng** through the oXBS and BS protocols to ensure you have enough DNA for optimization purposes.

The samples should be finally made up to **200 µl** after BS or oXBS conversion.

2 µl - **8 µl** can be used for each qMSP reaction depending on starting concentration of DNA.

Once DNA has been oXBS converted and BS converted it can be stored at -20C until further analysis.

Quantitative Methyl Specific PCR

6

Preperation of standard curve for % Methylation

etermine the specificity of the primers to amplify methylated DNA in an abundance of unmethylated and vice versa. Converted DNA can be prepared according to the following volumes for 1 qMSP reaction: in unmeth0

% Methylation	FM (µL)	FU (µL)
100	10	0
30	3	7
9	0.9	9.1
2.7	0.27	9.73
0.8	0.08	9.92

NOTE: These values are for 1 reaction and should be multiplied by the number of technical replicates and sets of primers required.
Alternatively, serial dilutions can be prepared by diluting the fully methylated in unmethylated by a third (1/3) for each dilution.

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an

6.1 qMSP is performed according to the following for 1 reaction:

Reagent:	µL
iTaq Universal SYBR Green Supermix	10
Forward Primer (10µM)	1 (final concentration 100-500nM: varies depending on optimisation)
Reverse Primer (10µM)	1 (final concentration 100-500nM: varies depending on optimisation)
Template	2-8
Nuclease free water	make up to final volume
Final volume	20

NOTE: For each sample you will need to prepare 6x the reactions. Since 1 sample (in parallel) is BS converted and oXBS converted to calculate 5hmC by difference and triplicate technical replicates are the minimum suggested to ensure reproducibility of reactions.

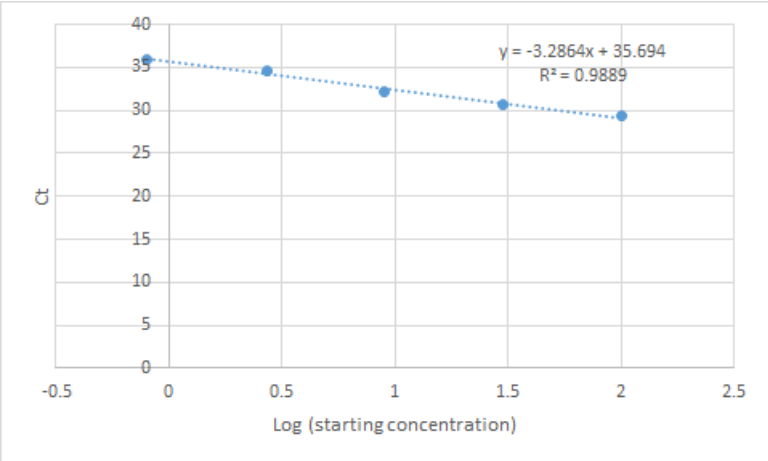
6.2 Amplify DNA according to the following qPCR protocol:

Stage	**Temperature (°C)**	**Time (min)**	
1	95	15	
2	95	0.15	
3	60	0.3	goto 2 x 40 times
4	55-95, 0.5°C increments	Melt Curve	
5	END		

6.3 EXPECTED RESULT FOR FULLY METHYLATED STANDARD CURVE

Plot the values obtained for each concentration of DNA (Ct) against the log(starting concentration). This should be conducted for each set of primers to determine efficacy.

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an



Expected result: Plot of the log (starting concentration) against Ct. This is important for determining the primer efficacy for amplifying methylated DNA target regions in the presence of unmethylated DNA.

NOTE: Plotted values from standard curve should yield a straight line with a high R2 and a slope of approximately 3.2 for both the methylated and unmethylated primers. If this is not the case, new primers should be designed or optimized further for DNA and primer concentrations and annealing temperatures.

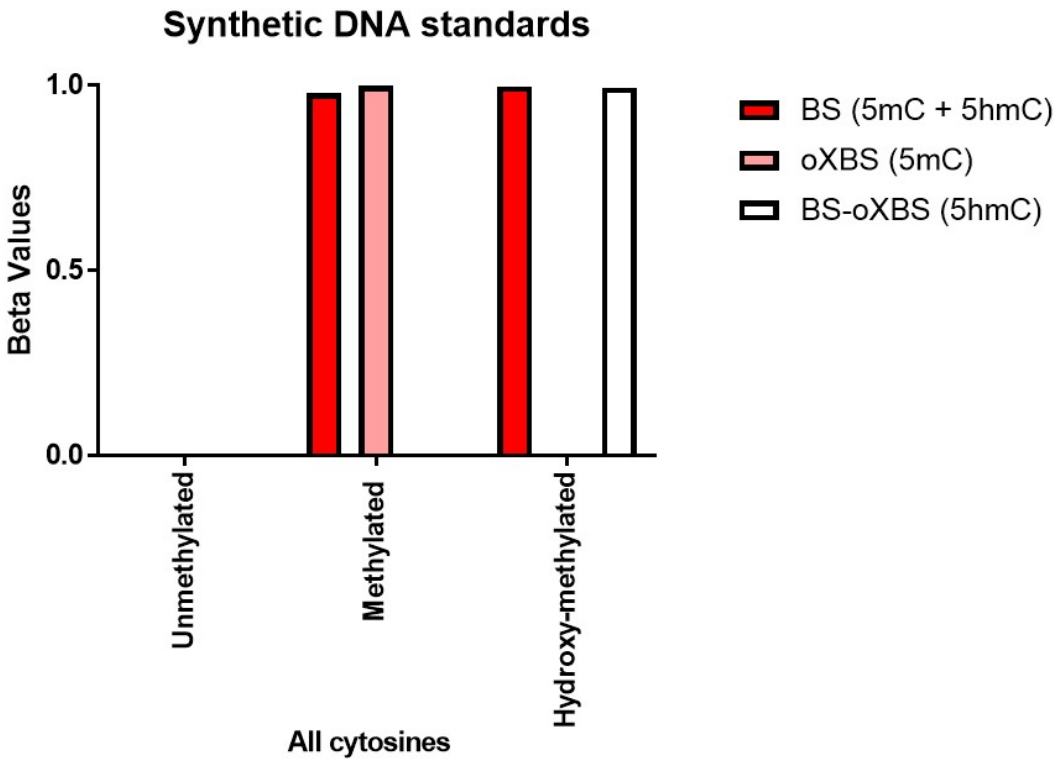
7 *Calculation of Beta Values*

The obtained Ct values can be used to calculate the % methylation at each loci according to the following analysis for the calculation of Beta Values:

2^- (CT values obtained by qPCR for Methylated primers) = M
2^- (CT values obtained by qPCR for Unmethylated primers) = U
Beta Values = M/(M+U)
5mC = Beta Value for oXBS
5hmC = Beta Value for BS – Beta Value for oXBS

Note: Beta Values Should be a value between 0 and 1. Negative Beta Values can be considered as 0.

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an



Synthetic DNA strands where every cytosine is either methylated, unmethylated or hydroxymethylated.

Expected result for synthetic DNA after oXBS-qMSP analysis.
Graphical representation of Beta Values obtained from synthetic DNA standards where all cytosine's in the sequence are either unmethylated, methylated or hydroxyl-methylated. Bisulfite converted DNA represents the combination of methylated and hydroxymethylated cytosines (red bar), while oxidative bisulfite represents the true amount of methylated cytosine's (pink bar), hence the hydroxyl-methylated cytosine's can be deduced by difference between BS and oXBS (white bar).

Citation: Hector Hernandez-Vargas, Chloe Goldsmith (09/07/2020). Quantitative analysis of methylation and hydroxymethylation using oXBS-qMSP. https://dx.doi.org/10.17504/protocols.io.52bg8an