BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit

Base Catalog # P-1067

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit is designed to identify loci- or gene-specific methylation and hydroxymethylation in any DNA regions. The optimized protocol and components of the kit allow subnanogram DNA to be used for DNA conversion by the combined bisulfite and cytosine deaminase treatment followed by qPCR in less than 5 hours.

Starting Material and Input amount: Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissue, cultured cells from a flask or microplate, microdissection samples, paraffin-embedded tissue, biopsy, embryonic cells, plasma/serum samples, and body fluid samples, etc. DNA enriched from various enrichment reactions such as ChIP, MeDIP/hMeDIP, or exon capture may also be used as starting material. The input amount of DNA can be from 10 ng to 200 ng. For optimal preparation, the input amount should be 50-100 ng.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the tube/vials. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

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KIT CONTENTS

Components		P-1067-48 2x 24 Reactions	Storage Temp
CNBF	Conversion Buffer	10 ml	RT
CVRP	Conversion Reagent	6 Vials	RT
DNBB	DNA Binding Buffer	15 ml	RT
NAOH	Denaturation Solution	100 µl	RT
DNAE	Modified DNA Elution	2 ml	RT
Clum	F-Spin Column	50	RT
Tube	F-Collection Tube	50	RT
APOE	APOBEC Enzyme	24 µl	-20°C
APOB	10 X APOBEC Reaction buffer	100 µl	-20°C
MMIX	Master Mix	1 ml	-20°C
MCST	Methylated DNA (500 ng/ml)*	5 µl	-20°C
HMCS	Hydroxymethylated DNA (500 ng/ml)*	5 µl	-20°C
MPRF	M-Control Primer-F (20 µM)*	10 µl	-20°C
MPRR	M-Control Primer-R (20 µM)*	10 µl	-20°C
UNPF	U-Control Primer-F (20 µM)*	10 µl	-20°C
UNPR	U-Control Primer-R (20 µM)*	10 µl	-20°C
AHDB	AH Dilution Buffer	4 ml	RT

** Spin the solution down to the bottom prior to use.*

SHIPPING & STORAGE

The kit is shipped in two parts: one part at ambient room temperature, and the second part on frozen ice packs at 4°C.

Upon receipt, store the components according to the temperatures in the table above away from light. The kit is stable for up to 6 months from the shipment date, when stored properly.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Vortex mixer
- ☐ Thermocycler
- ☐ Centrifuge including desktop centrifuge (up to 14,000 rpm)
- ☐ Pipettes and pipette tips

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- ☐ PCR tubes or plates
- ☐ 1.5 ml microcentrifuge tubes
- ☐ 100% Ethanol
- ☐ DNA/RNA-free Water
- ☐ DNA sample

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine (5mC). In somatic cells, 5mC is found almost exclusively in the context of paired symmetrical methylation of the dinucleotide CpG, whereas in embryonic stem (ES) cells, a substantial amount of 5mC is also observed in non-CpG contexts. The biological importance of 5mC as a major epigenetic modification in phenotype and gene expression has been widely recognized.

5-hydroxymethylcytosine (5hmC), as a sixth DNA base with functions in transcription regulation, has been detected to be abundant in human and mouse brains and embryonic stem (ES) cells. In mammals, it can be generated by the oxidation of 5mC, a reaction mediated by the ten-eleven translocation (TET) family of 5mC-hydroxylases.

5hmC has been demonstrated to be tissue specific, ranging from undetectable levels in cultured cell lines to 0.6% in human brain tissues, and can be as high as 8% of total DNA in some other species. The biological

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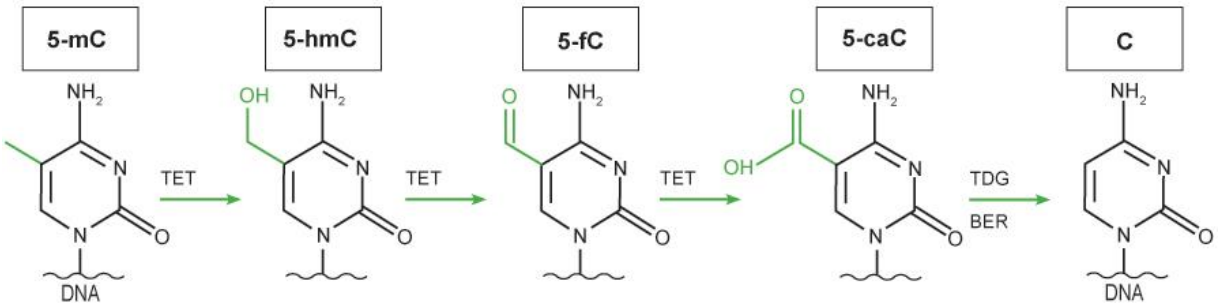
significance of 5hmC as an important epigenetic modification in phenotype and gene expression has been recently recognized. For example, global decrease in 5hmC content (DNA hypohydroxymethylation) has been exhibited in nearly all cancers and has been proposed as a molecular marker and therapeutic target in cancer as well.



More importantly, in contrast to 5mC that is generally considered as a gene repression marker, 5hmC is regarded as an intermediate of DNA demethylation and associated with expressed genes with a potential role in activating genes. Like 5mC, 5hmC remains the same after bisulfite-conversion of cytosine to uracil (C to U), which makes 5hmC indistinguishable from 5mC. As consequence, all 5hmC will be misread as 5mC in the assay using MS-PCR or bisulfite-seq. Therefore, the result is incorrect because DNA methylation status includes both 5mC and 5hmC.

Currently, several methods such as TET-assisted bisulfite sequencing (TAB-Sec) and oxidative bisulfite sequencing (oxBS-seq) are used for genome-wide 5hmC mapping at single base-resolution. However, they can be costly and time-consuming. Because few techniques exist for loci or gene-specific detection of 5mC & 5hmC, EpigenTek has addressed this problem with the development of the BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit.

This kit has the following features:

- **Simultaneous Reading:** DNA treatment is prepared simultaneously for both 5hmC and 5mC, which allows for parallel identification in a loci- or gene-specific manner simultaneously by qPCR.
- **Innovative Method:** A combination of bisulfite and subsequent cytosine deaminase (APOBEC) treatment allows for tactical conversion of 5mC in order to identify 5hmC.
- **High Specificity:** 5hmC is discriminated from C and 5mC as well as from other modified cytosines 5fC and 5caC.
- **Flexibility:** Perform 24 reactions for 5hmC and 24 reactions for 5mC analysis simultaneously, or perform 24 reactions for just 5hmC or 48 reactions for 5mC.
- **Fast and Streamlined Procedure:** The procedure from DNA bisulfite treatment to PCR products can be completed within 5 hours.
- **Complete Conversion:** Completely converts C into uracil (>99%) and 5mC to thymine (>95%)
- **Broad sample suitability**: Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissue, cultured cells from a flask or microplate, microdissection samples, paraffin-embedded tissue, biopsy, embryonic cells, plasma/serum samples, and body fluid samples, etc. DNA enriched from various enrichment reactions such as ChIP, MeDIP/hMeDIP, or exon capture may also be used as starting material.

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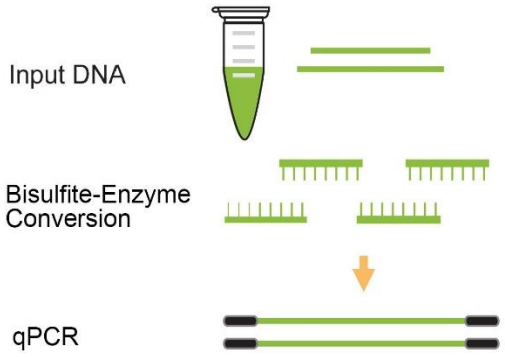
PRINCIPLE & PROCEDURE

This kit includes all reagents required for successful qPCR using converted DNA generated from a tiny amount of input DNA. In this preparation, DNA is simultaneously bisulfite modified and fragmented to the appropriate length during the bisulfite process. During the bisulfite treatment, unmodified cytosine (C) is converted to uracil and will be read as T in the sequencing. 5-methylcytosine (5mC) remains the same and 5-hydroxymethylcytosine (5hmC) forms cytosine 5-methylenesulfonate (CMS). The bisulfite modified DNA is further treated with specific APOBEC deaminase, which converts 5mC to thymine but not affect CMS. During the PCR and sequencing, CMS will still be read as C so that the 5hmC can be discriminated not only from C, 5mC but also other modified cytosines such as 5fC and 5caC (see the table 1). The bisulfite-enzyme converted DNA can then be used for qPCR for loci specific detection of 5mC and 5hmC.

Table 1: Principle of BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit

		C	5mC	5hmC	5fC	5caC
Reference		C	C	C	C	C
BisulPlus	Bisulfite Conversion	T	C	C	T	T
	APOBEC Treatment	T	T	C	T	T



◄ ***Fig 1***. *Workflow of the BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit.*

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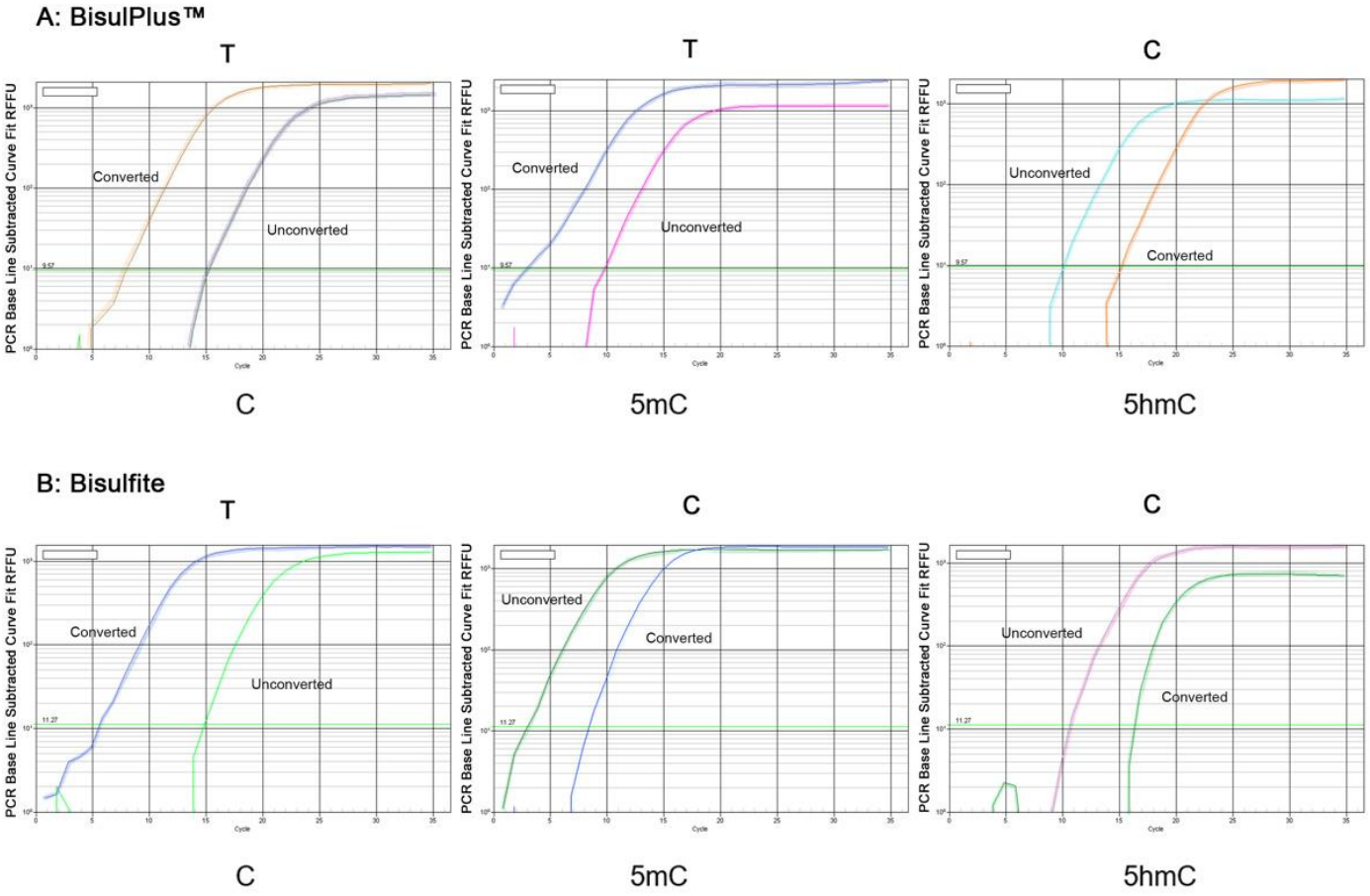

▲ ***Fig 2.*** *Discrimination of 5hmC from cytosine and 5mC by qPCR: Unmodified, methylated and hydroxymethylated DNA standards are used for bisulfite-enzyme treatment using the BisulPlus™ Loci 5mC & 5hmC Detection PCR Kit followed by real time amplification with use of primers targeting to CpG converted or unconverted sequences: A: BisulPlus™; B: Bisulfite standalone. Amplification lines are highlighted for visibility.*

ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Input DNA Amount: DNA amount can range from 2 ng to 200 ng per reaction. An optimal amount is 10 ng-100 ng per reaction. Starting DNA may be in water or in a buffer such as TE. DNA should be high quality and relatively free of RNA. RNase I can be used to remove RNA.

DNA Isolation: You can use your method of choice for DNA isolation. EpigenTek offers a series of genomic DNA isolation kits for your convenience.

DNA Storage: Isolated genomic DNA can be stored at 4°C or -20°C until use.

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1. Bisulfite DNA Modification

a. Add 1 ml of **Conversion Buffer** to 1 vial of **Conversion Reagent** to generate **Modification Solution**. Mix by inverting and shaking the vial repeatedly for 3-4 min (a trace amount of undissolved **Conversion Reagent** may remain, which is normal as **Conversion Reagent** is saturated in solution).

b. For each 0.2 ml PCR tube, add 150 µl of the mixed **Modification Solution** followed by adding 1-10 µl of sample DNA (20-100 ng) or 1-2 µl of methylated (MCST) and hydroxymethylated (HMCS) control DNA.

Note: *Check if the sample DNA volume is large and if the concentration is less than 5 ng/µl. If so, it is recommended to concentrate DNA using EpigenTek's DNA Concentrator Kit (Cat. No. P-1006) prior to bisulfite treatment.*

*Prepared **Modification Solution** can be stored at -20°C for up to 2 weeks without significant loss of efficiency. For the best results, the mixed solution should be used immediately.*

c. Tightly close the PCR tubes and place them in a thermocycler with heated lid. Program and run the thermocycler according to the following:

95°C 30 sec
65°C 45 min
95°C 30 sec
65°C 45 min
Hold 18-20°C up to 6 h

Meanwhile, insert the number of **F-Spin Columns** into **F-Collection Tubes** as needed by your experiment.

2. Converted DNA Clean-Up

a. Add 300 µl of **DNA Binding Buffer** to each column. Then transfer the samples from each PCR tube (from Step 1) to each column containing the **DNA Binding Buffer**. Centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

b. Add 250 µl of *90% ethanol* to each column. Centrifuge at 12,000 rpm for 45 sec.

c. Prepare final denaturation buffer by adding 10 µl of **Denaturation Solution** to every 1 ml of *90% ethanol*, and mix. Add 100 µl of the final denaturation buffer (**Denaturation Solution** and *90% ethanol* mixture) to each column. Allow columns to sit for 10 min at room temperature, then centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

d. Add 250 µl of *90% ethanol* to each column. Centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes. Add 250 µl of *90% ethanol* to each column again and centrifuge at 12,000 rpm for 45 sec.

e. Insert each column into a new 1.5 ml tube. Add 12.5 µl of **Modified DNA Elution** directly to each column's filter membrane. Centrifuge at 12,000 rpm for 60 sec to elute converted DNA.

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3. Enzyme DNA Conversion

a. Prepare enzyme DNA Conversion reaction in 0.2 ml PCR tube according to Table 2.

Table 2 Enzyme DNA Conversion

Component	Volume
Bisulfte DNA (from Step 2)	12 µl (10-50 ng input DNA)
10 X APOBEC Reaction Buffer	2 µl
APOBEC Enzyme	1 µl
AH Dilution Buffer	5 µl
Total volume	**20 µl**

Note: *A master mix of the reaction components can be prepared if multiple reactions are processed.*

b. Mix and incubate for 90 min at 37°C in a thermocycler without heated lid (make sure to set lid temperature to 25°C) followed by 72°C for 10 min.

Note: *After this step, the samples can be stored at 4°C overnight.*

4. Real Time PCR Analysis

1. *Primer Design*

   a) The primers designed should meet the criteria for real time PCR. For example, the covered sequence region should be 50-150 bp in length. G/C stretches at 3' ends of primers should be avoided.

   b) The primers designed can use the same criteria for Bisulfite conversion-based methylation specific PCR (MSP) or bisulfite genomic sequencing PCR (BSP).

2. *Prepare the PCR Reactions*

   a) Thaw all reaction components including **Master Mix**, DNA/RNA-free Water, **Primer solutions**, **Methylated DNA, Hydroxymethylated DNA** and DNA template. Mix well by vortexing briefly. Keep components on ice while in use, and return to –20°C immediately following use.

   b) Add components into each well according to the following procedures:

Component	Size (µl)	Final Concentration
Master Mix (2x)	10 µl	1X
Forward Primer	1 µl	0.4-0.5 µM
Reverse Primer	1 µl	0.4-0.5 µM
DNA Template	1-2 µl	100 pg-0.1 µg
DNA/RNA-free H_2O	6-7 µl	
Total Volume	**20 µl**	

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Note: *For the Negative Control, use DNA/RNA-free water instead of DNA template.*

3. *Program the PCR Reactions*

   a) Place the reaction plate in the instrument.

   b) Set the PCR conditions as follows:

Cycle Step	Temperature	Time	Cycle
Activation	95°C	60 sec	1
Cycling	95°C 60°C	15 sec 30 sec	40-45
Melt Cure	60-95°C	various	1

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
DNA is poorly converted	Poor DNA quality (DNA is severely degraded).	Check if the sample DNA 260/280 ratio is between 1.8-1.9 and if DNA is degraded by running gel. Ensure that RNA is removed by RNase treatment.
	Too little DNA or too much DNA (i.e., <5 pg or >200 ng).	Increase or decrease input DNA to within the correct range, or to the optimal amount of 50-100 ng.
	Temperature or thermal cycling condition is incorrect.	Check for appropriate temperature or thermal cycling conditions.
	Added amount of conversion reagents including bisulfite and **APOBEC enzyme** is not sufficient.	Ensure that sufficient conversion reagents are is added into the reaction.
Elute contains little or no DNA	Poor input DNA quality (degraded).	Check if DNA is degraded by running a gel.
	DNA Binding Buffer is not added into the sample.	Ensure that **DNA Binding Buffer** is added in Step 2a.
	Concentration of ethanol solution used for DNA clean-up is not correct.	Use *90% ethanol* for DNA clean-up.
	Sample is not completely passed through the filter membrane of column.	Centrifuge for 1 min at 12,000 rpm or until the entire sample has passed through the filter membrane.
Little or No Amplification Product	Primer is incorrect.	Check primer design. If it is specifically for bisulfite-treated DNA, confirm the accuracy of the sequence information. Redesign the primers according to the guidance of the MS-PCR primer design or by using MethPrimer.

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	Primer is degraded or primer concentration is not optimal.	Repeat PCR with different primer concentration by 0.1 µM increments. Check for possible degradation of the primers on a denaturing polyacrylamide gel.
	Incorrect PCR program including insufficient denaturing, annealing & extension time/temperature, and insufficient number of cycles.	Check if the denaturing, annealing, and extension time/temperature programming is correct. Increase number of cycles.
Non-Specific Amplification	Template concentration is too high.	When amplifying bisulfite DNA, the initial concentration of template in the reaction mixture should not exceed 100 ng per 20 µl of reaction volume.
	Insufficient conversion of DNA.	Check PCR specificity and reliability with β-actin primer included in the kit. If necessary, make new converted DNA and repeat PCR.
	Primer design is not optimal.	Check primer design. If it is specifically for bisulfite-treated DNA, confirm the accuracy of the sequence in formation. Redesign the primers according to the guidance of the MS-PCR primer design or by using MethPrimer.

RELATED PRODUCTS

DNA Isolation and Cleanup

P-1003	FitAmp™ General Tissue Section DNA Isolation Kit
P-1004	FitAmp™ Plasma/Serum DNA Isolation Kit
P-1006	DNA Concentrator Kit
P-1009	FitAmp™ Paraffin Tissue Section DNA Isolation Kit
P-1017	FitAmp™ Urine DNA Isolation Kit
Q10002	EpiMag HT (96-Well) Magnetic Separator
P-1018	FitAmp™ Blood and Cultured Cell DNA Extraction Kit

DNA Bisulfite Conversion

P-1001	Methylamp™ DNA Modification Kit
P-1026	BisulFlash™ DNA Modification Kit

PCR Analysis

P-1028	Methylamp MS-qPCR Fast Kit

DNA Library Preparation

P-1051	EpiNext™ DNA Library Preparation Kit (Illumina)
P-1053	EpiNext™ High-Sensitivity DNA Library Preparation Kit (Illumina)
P-1056A	EpiNext™ High-Sensitivity Bisulfite-Seq Kit Kit
P-1059	EpiNext™ DNA Size Selection Kit
P-1063	EpiNext™ DNA Purification HT System

NGS Barcode

P-1060	EpiNext™ NGS Barcode (Index) Set-12

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