# AmoyDx® MLH1 Methylation PCR Kit

Instructions for Use

For Research Use Only. Not for use in diagnostic procedures.

REF 8.01.0293 24 tests/kit For LightCycler480 II, cobas z 480, QuantStuido5, ABI7500, SLAN-96S

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## Background

MLH1 is a gene responsible for encoding a protein crucial for DNA repair and maintaining genome stability. The MLH1 protein plays a vital role in the DNA mismatch repair system, correcting mismatches and insertion/deletion errors in DNA. Methylation of the MLH1 gene involves an epigenetic modification, where methyl groups interact with specific regions of the DNA molecule, influencing gene expression and the production of the MLH1 protein. This methylation is of significant interest in cancer research, particularly in the context of tumors such as colorectal cancer. Aberrant MLH1 gene methylation may lead to suppressed MLH1 protein production, disrupting the normal function of the DNA repair system and increasing the risk of cellular mutations, thereby promoting tumorigenesis. The assessment of MLH1 methylation status is a critical aspect of cancer diagnostics, serving as a biomarker to comprehend tumor development and guide treatment directions. A comprehensive understanding of MLH1 methylation contributes to unraveling the molecular mechanisms of cancer and advancing targeted therapeutic interventions.

## Intended Use

The AmoyDx® MLH1 Methylation PCR Kit is a real-time PCR assay for the qualitative detection of DNA methylation is MLH1 gene in human genomic DNA extracted from human colorectal cancer formalin-fixed paraffin-embedded (FFPE) tissue.

The kit is for research use only and intended to be used by trained professionals in a laboratory environment.

## Principles of the Procedure

The kit adopts real-time PCR technology which uses specific primers and fluorescent probes for analysis methylation status of MLH1 gene in human DNA samples. The bisulfite-treated methylated sequence in DNA samples are amplified by MLH1 methylation specific primers, and then the amplicon is detected by fluorescent probes labeled with FAM to detect methylated CpG sites in MLH1 gene. The internal control system is included in MLH1 methylation detection system to assess DNA quality and monitor the whole PCR reaction process.

The kit comprises MLH1 Reaction Mix, Enzyme Mix, and Positive Control. The MLH1 Reaction Mix is designed to detect methylated fragments of the MLH1 gene, concurrently containing the internal control gene GAPDH to monitor Sample DNA quality and the PCR reaction process. The methylation signal of the MLH1 gene is indicated by FAM, while the GAPDH gene signal is indicated by VIC/HEX.

## Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

| Content | Main Ingredients | Quantity |
|---|---|---|
| **MLH1 Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 µL/tube ×1 |
| **MLH1 Enzyme Mix** | Taq DNA Polymerase | 22 µL/tube ×1 |
| **MLH1 Positive Control** | Plasmid DNA | 200 µL /tube ×1 |

***Note:***

*Do not mix reagents from different batches.*

## Storage and Stability

The kit requires shipment on frozen ice packs below 25℃ for no more than one week. All contents of the kit should be stored immediately upon receipt at -20±5℃ and protected from light.

The shelf-life of the kit is twelve months. Tube opening doesn't affect expiration of the kit. The recommend maximum freeze-thaw cycle is five cycles.

## Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:
   LightCycler480 II, cobas z 480, QuantStuido5, ABI7500, SLAN-96S
2) DNA extraction kit: we recommend use of AmoyDx® FFPE DNA Kit, for paraffin embedded specimens.
3) DNA bisulfite treatment kit: we recommend use of Qiagen Epitect Fast DNA Bisulfite Kit (Catalog no. 59824)
4) Spectrophotometer for measuring DNA concentration.
5) Mini centrifuge with rotor for centrifuge tubes.
6) Mini centrifuge with rotor for PCR tubes.
7) Vortexer.
8) Nuclease-free centrifuge tubes.
9) Nuclease-free PCR tubes and caps.
10) Adjustable pipettors and filtered pipette tips for handling DNA.
11) Tube racks.
12) Disposable powder-free gloves.
13) Sterile, nuclease-free water.

## Precautions and Handling Requirements

### Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use, and strictly follow the instruction during operation.
- Please check the compatible real-time PCR instruments prior to use.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT use any other reagents from different lots in the tests.
- DO NOT use any other reagent in the other test kits.

### Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- Only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.





- Avoid skin, eyes and mucous membranes contact with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipet by mouth.

### Decontamination and Disposal

- The kit contains positive control; strictly distinguish the positive control from other reagents to avoid contamination which may cause false positive.
- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Using separate, dedicated pipettes and filtered pipette tips when handling samples and reagents to prevent exogenous nucleic acid contamination to the reagents.
- Please pack the post-amplification tubes with two disposable gloves and discard properly. DO NOT open the post- amplification PCR tubes.
- All disposable materials are for one time use. DO NOT reuse.
- The unused reagents, used kit, and waste must be disposed of properly.

### Cleaning

- After the experiment, wipe down the work area, spray down the pipettes and equipment with 75% ethanol or 10% hypochlorous acid solution.

### Instrument Setup

- Setup the reaction volume as 40 μL.
- For SLAN-96S instrument, please set up as follows: Fluorophores/Dyes: FAM, VIC. During the result interpretation, select "Selected Wells" for "Y-Axis Scaling Auto-adjust By" and "Absolute fluorescence Method" for "Normalization algorithm".
- For QuantStuido5 and ABI7500 instrument, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: None; Passive Reference: None.
- Refer to the real-time PCR instrument operator's manual for detailed instructions.
- We recommend that for all PCR instruments in use, a fluorescence calibration should be conducted once a year.

## Assay Procedure

### 1. DNA Extraction and Bisulfite Treatment

The specimen material must be extracted DNA from FFPE tissue samples. DNA extraction kit and bisulfite treatment kit are not included in the kit. Before DNA extraction, it's essential to use standard pathology methodology to ensure tumor sample quality. It's better to use tumor tissue samples with more than 20% tumor cells. Carry out the DNA extraction and bisulfite treatment according to the instructions of DNA extraction kit and bisulfite treatment kit. The original extracted and bisulfite-treated DNA should be directly used for MLH1

methylation PCR testing without dilution.

The OD value and concentration of extracted DNA should be measured using the spectrophotometer after extraction. The $OD_{260}/OD_{280}$ value should be between 1.5~2.2. The DNA concentration should be more than 10 ng/μL. If the DNA concentration and OD value do not meet the requirements, it is recommended to consider re-sampling or increasing the sample quantity before proceeding with DNA extraction.

***Note:***

- *The FFPE tissue should be handled and stored properly. The storage time should preferably be less than 3 years.*
- *It's recommended to use ≥ 5 FFPE tissue slides with a thickness of 5 μm.*
- *The extracted DNA should be used for bisulfite treatment immediately. If not, it should be stored at -20±5℃ for no more than 6 months.*
- *The bisulfite-treatment DNA should be used for PCR testing immediately. If not, it should be stored at -20±5℃ for no more than 3 months.*

**2. Methylation PCR Detection**

In each PCR process, each sample must be tested and analyzed together with a **Positive Control (PC)** and a **No Template Control (NTC, self-prepared purified water)**.

1) Take the **MLH1 Reaction Mix** and **Positive Control (PC)** out of the kit from the freezer, and **Enzyme Mix** remained in freezer at -20±5℃.
2) Thaw the **MLH1 Reaction Mix** and **Positive Control (PC)** at room temperature. When the reagents are completely thawed, mix each reagent thoroughly by vortexing and centrifuge for 15 seconds to collect all liquid at the bottom of the tube.
3) Take the **Enzyme Mix** out and centrifuge for 15 seconds prior to use.
4) Prepare an adequate MLH1 Master Mix in a sterile centrifuge tube based on the total number of reactions needed for DNA samples, Positive Control (PC), and No Template Control (NTC). The MLH1 Master Mix should include MLH1 Reaction Mix and Enzyme Mix in accordance with the ratios specified in Table 2. Thoroughly mix the MLH1 Master Mix using vortexing and then centrifuge for 15 seconds.

Table 2 MLH1 Master Mix

| Content | Volume per test |
|---|---|
| **MLH1 Reaction Mix** | 35 μL |
| **MLH1 Enzyme Mix** | 0.3 μL |
| **Total Volume** | 35.3 |

***Note:***

- *Each run must contain one PC and one NTC.*
- *The prepared mixtures should be used immediately, avoid prolonged storage.*
- *Due to the viscosity of the enzyme mix, pipet slowly to ensure all mix is completely dispensed from the tip.*





- *Pipet enzyme mix by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.*

5) Dispense 35 μL of the MLH1 Master Mix into each PCR reaction tube, following the layout provided in Table 3 as a reference.
6) Take out the bisulfite-treated Sample DNA and nuclease-free water for No Template Control (NTC) and Positive Control (PC).
7) Add 5 μL of the Sample DNA, 5 μL of No Template Control (NTC), and 5 μL of Positive Control (PC) into their respective PCR reaction tubes, following the layout in Table 3 as a reference. Cover the tubes with caps and centrifuge quickly for 15 seconds.
8) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
9) Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 3.

Table 3 PCR Plate Layout

| Well | 1 | 2 | 3 |
|---|---|---|---|
| **A** | Sample 1 | Sample 9 | Sample 17 |
| **B** | Sample 2 | Sample 10 | Sample 18 |
| **C** | Sample 3 | Sample 11 | Sample 19 |
| **D** | Sample 4 | Sample 12 | Sample 20 |
| **E** | Sample 5 | Sample 13 | Sample 21 |
| **F** | Sample 6 | Sample 14 | Sample 22 |
| **G** | Sample 7 | Sample 15 | NTC |
| **H** | Sample 8 | Sample 16 | PC |

10) Setup the PCR protocol using the cycling parameters in Table 4.

Table 4 Cycling Parameters

| Stage | Cycles | Temperature | Time | Data collection |
|---|---|---|---|---|
| 1 | 1 | 95℃ | 5 min | / |
| 2 | 10 | 95℃ | 25 s | / |
| | | 64℃ | 20 s | / |
| | | 72℃ | 20 s | / |
| 3 | 30 | 93℃ | 25 s | / |
| | | 60℃ | 35 s | FAM and VIC/HEX |
| | | 72℃ | 20 s | / |
| 4 | 1 | 40℃ | 30 s | / |

11) Start the PCR run immediately.
12) When the PCR run is finished, analyze the data according to the "Results Interpretation" procedures.

**3. Result Interpretation**

1) Select one reaction tube and one fluorescent signal at a time for data analysis.

***Before the data analysis, the following items should be checked:***

2) For No Template Control (NTC): In FAM channel, there should be no amplification curves. If there were amplification FAM channel, it means that there is pollution or operation error in the laboratory, and the test result is invalid.
3) For Positive Control (PC): The FAM and VIC/HEX Ct values should be <22, if not, the test is invalid. The sample should be retested.

***Analyze the methylation assay for each sample:***

4) Check the Internal Control for each sample:

   a) If the VIC/HEX Ct value is ≤22, continue with the analysis.

   b) If the VIC/HEX Ct value is >22, which indicates the DNA sample contains PCR inhibitors or DNA amount is insufficient. The sample should be retested with re-exacted or sufficient DNA.

5) Check the FAM Ct values of MLH1 Reaction Mix for each sample.

   a) Record the FAM Ct values of MLH1 Reaction Mix and calculate the ΔCt values.

   b) ΔCt value = FAM Ct value – Internal Control VIC/HEX Ct value

   c) If the ΔCt value is less than 5 (ΔCt < 5), it indicates that the sample contains MLH1 gene methylation.

   d) If the ΔCt value is greater than or equal to 5 (ΔCt ≥ 5), it indicates that the sample does not contain MLH1 gene methylation, or the methylation level is below the detection limit of the kit.

## Limitations

1) This kit is intended for use only by individuals who have received specialized training in PCR techniques.
2) The test results obtained with this kit are for research purposes only and should not be used for diagnostic procedures. When considering personalized treatment for patients, results should be interpreted comprehensively alongside their symptoms, medical history, other laboratory tests, and treatment responses.
3) The reliability of results is contingent on proper sample processing, transport, and storage.
4) The kit is designed to work exclusively with the specified specimen type, compatible real-time PCR instrument, and DNA extraction assay.

## Symbols

| | | | |
|---|---|---|---|
|  | Manufacturer |  | Catalogue Number |
|  | Batch Code |  | Use-by Date |
|  | Contains Sufficient for <n> Tests |  | Temperature Limit |
|  | Consult Instructions For Use |  | Keep Dry |
|  | This Way Up |  | Fragile, Handle With Care |
|  | Keep Away from Sunlight | | |