AmoyDx® Microsatellite Instability (MSI) Detection Kit

Real-time PCR with Melting Curve Analysis (MCA)

Instructions for Use

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

REF	8.01.0263	24 tests/kit	For LightCycler 480 II, cobas® z480
REF	8.01.0255	24 tests/kit	For SLAN-96S

Amoy Diagnostics Co., Ltd.
No. 39, Dingshan Road, Haicang District,
361027 Xiamen, P. R. China
Tel: +86 592 6806835
Fax: +86 592 6806839
E-mail: sales@amoydx.com
Website: www.amoydx.com

Version: P1.2
Aug 2023

Background

Microsatellites (MS) are short tandem repeats (STRs) of DNA sequences (1~6 bp, mainly dinucleotide or mononucleotide) that are widely spread throughout the genome, and also known as simple sequence repeats (SSRs) [1]. Microsatellites account for 3% of the genome [2]. The number of microsatellite repeats is the same in normal genome and is hereditary stable, which is referred to as microsatellite stability (MSS). Microsatellite instability (MSI) is the accumulation of replication errors at microsatellite sequences in case of deficient mismatch repair status [3].

According to the number of microsatellite instability loci, colorectal cancer (CRC) can be divided into three distinct phenotypes: microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L) and microsatellite stable. Defective mismatch repair (dMMR) in colorectal cancer can be equivalent to MSI-H, and proficient mismatch repair (pMMR) can be equivalent to MSI-L/MSS.

Studies reported that MSI frequency in CRC varies according to the stage of the disease, with a higher incidence in the early stages (~20% in stages I and II, and ~12% in stage III) and a lower incidence in the advanced metastatic setting (4%-5% in stage IV) [4-1]. Mismatch repair protein immunohistochemistry (IHC) detection and/or MSI DNA detection has become the main screening strategy for Lynch syndrome (LS), and more than 90% of LS patients have MSI[6]. The Chinese society of clinical oncology (CSCO) guidelines for the diagnosis and treatment of colorectal cancer recommend that colorectal cancer patients aged 70 years and below can undergo MMR protein IHC detection and/or MSI DNA detection (level two recommendation), dMMR/MSI-H patients were further tested for germline mutations of LS-related genes to confirm the diagnosis [7].

Intended Use

The AmoyDx® Microsatellite instability Detection Kit is an *in vitro*, melting curve analysis (MCA)-based real-time PCR assay for the qualitative detection of microsatellite instability status in eight mononucleotide markers (see Table 1) in DNA samples extracted from human colorectal cancer formalin-fixed paraffin-embedded (FFPE) tissue.

According to the number of microsatellite instability loci, this product divides colorectal cancer into two types of microsatellite status: high microsatellite instability and none-high microsatellite instability (MSI-L/MSS), and assists in the diagnosis of colorectal cancer with mismatch repair gene mutation, combined with other clinical results to assist clinicians in the diagnosis of Lynch syndrome in colorectal cancer, provides an evidence for early detection of colorectal cancer characterized by DNA mismatch repair defects.

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

Table 1 Markers Detected by the Assay

No.	MS Marker	Gene ID	Base Repeats	Change of Repeat Length (bp)
1	EIF4E3	317649	$(A)_{12}$	12→10
2	IFT140	9742	$(T)_{11}$	11→10
3	PPP1CC	5501	$(A)_{12}$	12→10
4	UBAC2	337867	$(T)_{12}$	12→10
5	PRR5-ARHGAP8	553158	$(T)_{12}$	12→10
6	ACVR2A	92	$(A)_{8}$	8→7
7	TAOK3	51347	$(A)_{12}$	12→10
8	RBM14-RBM4	100526737	$(T)_{12}$	12→10

Principles of the Procedure

The kit adopts real-time PCR melting curve analysis method to detect eight markers. By counting the number of microsatellite instability loci, it can be judged whether microsatellite instability occurs in the sample. The kit contains specific primers for each marker and specific FAM/CY5-labeled fluorescent probes for designated mutant sequences. When the PCR amplification is completed, carry out the melting curves analysis in PCR amplicon. When there is a microsatellite unstable template in the sample, a melting peak will be generated in a specific Tm value range, and the microsatellite status of the sample can be determined by judging the "presence/absence" of the marker mutant melting peak.

The reaction system includes specific primers and VIC signal-labeled fluorescent probes for internal control reference gene, to assess the sample quality, detect the presence of inhibitors and monitor the accuracy of the experimental operation. The use of uracil-N-glycosylase (UNG) and dUTP in the kit can hydrolyse the PCR amplicon containing dUTP to prevent PCR amplicon carryover contamination.

Kit Contents

This kit contains the following materials:

Table 2 Kit Contents

Content	Main Ingredients	Quantity
PCR MSI Reaction Mix	8-tube Strip*	12 strips
PCR MSI Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	190 µL/tube ×1
Dilution Buffer	Tris, EDTA	1800 µL/tube ×4
PCR MSI Negative Control	MSI Negative DNA	150 µL/tube ×1
PCR MSI Positive Control	MSI Positive Plasmid DNA	150 µL/tube ×1

* Each strip (8-tube) includes the following contents for testing of two samples or two controls (Table 3).

Table 3 Information of the 8-tube Strip

Tube No.	Reagent	Main Ingredients	Quantity	Fluorescent Signal		
				FAM	CY5	VIC
1/5	PCR MSI Reaction Mix 1	Primers, Probes, Mg^{2+}, dNTPs	33.5 µL	EIF4E3	IFT140	IC
2/6	PCR MSI Reaction Mix 2	Primers, Probes, Mg^{2+}, dNTPs	33.5 µL	PPP1CC	UBAC2	IC
3/7	PCR MSI Reaction Mix 3	Primers, Probes, Mg^{2+}, dNTPs	33.5 µL	PRR5-ARHGAP8	ACVR2A	IC
4/8	PCR MSI Reaction Mix 4	Primers, Probes, Mg^{2+}, dNTPs	33.5 µL	TAOK3	RBM14-RBM4	IC

* IC: Internal Control

Note: *Distinguish Tube ⑧ from Tube ① according to the hole position at the strip edge, described as follows.*

For LightCycler 480 II, cobas® z480:

For SLAN-96S:

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: SLAN-96S, LightCycler 480 II, or cobas® z480.
2) DNA extraction kit: we recommend use of AmoyDx DNA extraction kit (AmoyDx® FFPE DNA Kit, for paraffin embedded specimens).
3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Vortexer.
7) Nuclease-free centrifuge tubes.
8) Nuclease-free PCR tubes and caps.
9) Adjustable pipettors and filtered pipette tips for handling DNA.
10) Tube racks.
11) Disposable powder-free gloves.
12) 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, please set up as follows: in "General" window, select "Melting Curve" for "Project Type", in "Program" window, select "Continuous" and set up "Ramp Rate" as 0.02℃/s.
- For LightCycler480 II, in "Experiment" window, click "Customize" to pop up the dialog box "Detection Format", select "Dynamic" Mode, and Filter Combination as "465-610, 533-580, and 618-660". Select "Analysis Mode" as "Melting Curves" in Stages 3 and 4.
- For cobas® z480, in "Experiment" window, click "Customize" to pop up the dialog box "Detection Format", select "Dynamic" Mode, and Filter Combination as "465-610, 540-580, and 610-670". Select "Analysis Mode" as "Melting Curves" in Stages 3 and 4.
- 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

The specimen material must be extracted DNA from colorectal cancer FFPE tissue samples. DNA extraction kit is 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 according to the instructions of DNA extraction kit.

The OD value 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 used in the testing is recommended to be 5 ng/µL. If the extracted DNA is less than 5 ng/µL, the sample should be re-extracted. If the extracted DNA is more than 5 ng/µL, dilute the extracted DNA to 5 ng/µL with Dilution Buffer provided in the kit.

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 immediately. If not, store the original extracted DNA at -20±5℃ for no more than 6 months.*

2. MSI Detection

1) Take the **PCR MSI Negative Control (NC)**, **PCR MSI Positive Control (PC)** out of the kit from the freezer, and other reagents remained in freezer at -20±5℃.
2) Thaw the **PCR MSI Negative Control** and **PCR MSI Positive Control** at room temperature. When the reagents are completely thawed, mix each reagent thoroughly by vortexing and centrifuge for 5 seconds to collect all liquid at the bottom of the tube.
3) Take the **PCR MSI Enzyme Mix** out and centrifuge for 5 seconds prior to use.
4) Take out the sample DNA and nuclease-free water for No Template Control (NTC).
5) Prepare NTC mixture: pipet 22 μL nuclease-free water (NTC) and 6.6 μL **PCR MSI Enzyme Mix** into one centrifuge tube. Mix them thoroughly by vortexing, and then centrifuge for 5 seconds.
6) Prepare NC mixture: pipet 22 μL **PCR MSI Negative Control** and 6.6 μL **PCR MSI Enzyme Mix** into one centrifuge tube. Mix them thoroughly by vortexing, and then centrifuge for 5 seconds.
7) Prepare sample DNA mixture: pipet 22 μL each sample DNA (5 ng/μL) and 6.6 μL **PCR MSI Enzyme Mix** into one centrifuge tube. Mix them thoroughly by vortexing, and then centrifuge for 5 seconds.
8) Prepare positive control (PC) mixture: pipet 22 μL **PCR MSI Positive Control** and 6.6 μL **PCR MSI Enzyme Mix** into one centrifuge tube. Mix them thoroughly by vortexing, and then centrifuge for 5 seconds.

Note:

- *Each run must contain one PC, one NC 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.*

9) Take out the **PCR MSI Reaction Mix** (sufficient for samples, PC, NC and NTC) and centrifuge the strips if there are any droplets in the caps of the PCR tubes. Then gently uncover the caps prior to use.
10) Take four PCR tubes (Tubes ①~④/⑤~⑧) for NTC: add 6.5 μL of prepared NTC mixture to each PCR tube, and cap the PCR tubes.
11) Take four PCR tubes (Tubes ①~④/⑤~⑧) for NC: add 6.5 μL of prepared NC mixture to each PCR tube, and cap the PCR tubes.
12) Take four PCR tubes (Tubes ①~④/⑤~⑧) for each sample: add 6.5 μL of prepared sample DNA mixture to each PCR tube, and cap the PCR tubes.
13) Take four PCR tubes (Tubes ①~④/⑤~⑧) for PC: add 6.5 μL of prepared PC mixture to each PCR tube, and cap the PCR tubes.
14) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.

Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 PCR Plate Layout

Well	1	2	...	11	12
A	NTC	Sample 1	…	Sample 19	Sample 21

B	NTC	Sample 1	…	Sample 19	Sample 21
C	NTC	Sample 1	…	Sample 19	Sample 21
D	NTC	Sample 1	…	Sample 19	Sample 21
E	NC	Sample 2	…	Sample 20	PC
F	NC	Sample 2	…	Sample 20	PC
G	NC	Sample 2	…	Sample 20	PC
H	NC	Sample 2	…	Sample 20	PC

15) Setup the PCR protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5 min	/
2	60	95℃	5 s	/
		54℃	15 s	/
		72℃	10 s	/
3	1	95℃	1 min	/
		45℃	3 min	/
		35℃	1 min	/
4	1	35~58℃	/	Melting curve

16) Start the PCR run immediately.

17) When the PCR run and melting curve analysis is finished, analyze the data according to the “Results Interpretation” procedures.

3. Result Interpretation

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

2) For each PCR MSI Reaction Mix, the FAM/CY5/VIC melting temperature (Tm) reference range for eight mononucleotide markers and Internal Control is indicated in the Table 6:

Table 6 Reference range of melting peak Tm value (temperature) of FAM/CY5/VIC channel in each reaction tube

PCR MSI Reaction Mix	FAM Tm Range	CY5 Tm Range	VIC Tm Range
1/5	47.5℃~50.5℃	49.0℃~52.0℃	44.0℃~47.0℃
2/6	47.5℃~50.5℃	48.0℃~51.0℃	44.0℃~47.0℃
3/7	47.0℃~50.0℃	48.0℃~51.0℃	44.0℃~47.0℃
4/8	50.0℃~53.0℃	46.5℃~49.5℃	44.0℃~47.0℃

Note: *the above Tm range is based on SLAN-96S. In case of Tm variation, the actual Tm value shall prevail.*

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

3) For NTC: In FAM, CY5, and VIC channels, there should be no melting peaks in the 4 reaction tubes in the reference range of Tm values (see Table 6). If there were melting peaks in VIC channel occasionally, and none of the 8 markers have melting peaks in the reference range of Tm values, it will not affect the interpretation of the test results. When the FAM, CY5 and VIC channels do not meet the above requirements, it means that there is pollution or operation error in the laboratory, and the test result is invalid.

4) For PCR MSI Negative Control (NC): In VIC channel, there should be melting peaks in the 4 reaction tubes in the reference range of Tm values. In FAM, CY5 channels, there should be no melting peaks in the 4 reaction tubes in the reference range of Tm values.

5) For PCR MSI Positive Control (PC): In VIC channel, there should be melting peaks in the 4 reaction tubes in the reference range of Tm values. In FAM, CY5 channels, there should be melting peaks for the eight markers in the 4 reaction tubes in the reference range of Tm values.

Analyze the MSI assay for each sample:

6) Check the Internal Control VIC signals of PCR MSI Reaction Mix 1~4 for each sample:

   a) If all the VIC signals of PCR MSI Reaction Mix 1~4 show melting peak in the reference range of Tm values, continue with the analysis.

   b) If any VIC signal of PCR MSI Reaction Mix 1~4 shows no melting peak in the reference range of Tm values, which indicates the DNA sample contains PCR inhibitors or DNA amount is insufficient. The sample should be retested with re-exacted or sufficient DNA.

7) Check the FAM/CY5 signals of PCR MSI Reaction Mix 1~4 for each sample.

   a) Record the number of melting peak in the reference range of Tm values in FAM/CY5 signals for eight markers, and interpret the result for each sample (see Table 7).

   b) If there is 0 or 1 melting peak in the reference range of Tm values in FAM/CY5 signal, the result should be determined as none-high microsatellite instability (MSI-L/MSS).

   c) If there are ≥ 2 melting peaks in the reference range of Tm values, the result should be determined as high microsatellite instability (MSI-H).

Table 7 Result interpretation

Number of melting peak for 8 markers (FAM/CY5)	Result
0 or 1	None-high microsatellite instability (MSI-L/MSS)
≥ 2	High microsatellite instability (MSI-H)

8) Examples of melting curve for different results are listed in Figure 1.

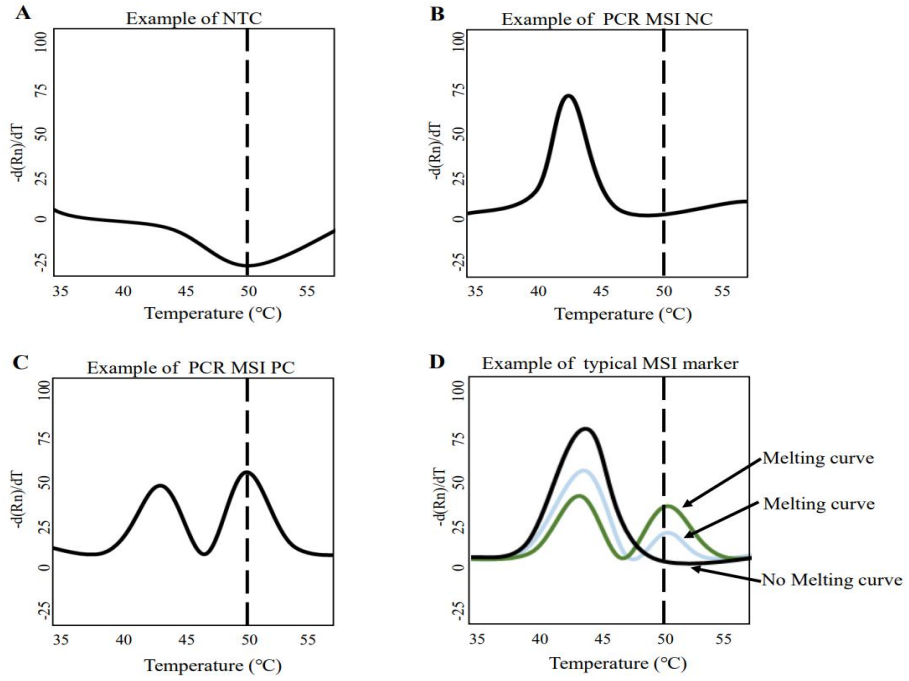

Figure 1 Examples of melting curve for different results

Performance Characterizes

1) Accuracy: Test Positive References, all results are positive and the concordance rate is 100%.

2) Specificity: Test Negative References, all results are negative and the concordance rate is 100%.

3) Limit of Detection: Test Sensitivity References for 3 repeats, all results are Microsatellite Instability (MSI).

4) Precision: Test Precision References for 10 repeats, the concordance rate is 100%.

Limitations

1) The kit is to be used only by personnel specially trained with PCR techniques.

2) The test results of this kit are for clinical reference only, and the selection of personalized treatment for patients should be considered comprehensively in combination with their symptoms, signs, medical history, other laboratory tests, and treatment responses.

3) When the test result is none-high microsatellite instability (MSI-L/MSS), the possibility of high microsatellite instability (MSI-H) cannot be completely ruled out. Insufficient tumor DNA in the sample, severe degradation or mutation allele frequency below the limit of detection can also cause none-high microsatellite instability (MSI-L/MSS) result.

4) Reliable results are dependent on proper sample processing, transport, and storage.

5) The kit can only work with the specified specimen type, compatible real-time PCR instrument and DNA extraction assay.

6) The kit can only detect the eight markers (See Table 1), excluding markers that are not declared in the detection kit.

References

1) Rajwant K K, Manoj K R, Sanjay K. Microsatellite markers: an overview of the recent progress in plants[J]. Euphytica, 2011, 177: 309–334.

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3) George P, Ian M F, Mark J. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome[J]. Histopathology, 2010, 56(2): 167-179.

4) Hassan A, Sadhna A, et al. A meta-analysis of MSI frequency and race in colorectal cancer[J]. Oncotarget, 2016, 7(23): 34546–34557.

5) Francesca B, et al. Microsatellite Instability in Colorectal Cancer: Overview of Its Clinical Significance and Novel Perspectives[J]. Clin Adv Hematol Oncol, 2018, 16(11):735-745.

6) Lynch H T, De la Chapelle A. Hereditary colorectal cancer[J]. New England Journal of Medicine, 2003, 348(10): 919-932.

7) Chinese Society of Clinical Oncology Guidelines Working Committee. Chinese Society of Clinical Oncology (CSCO) Guidelines for the Diagnosis and Treatment of Colorectal Cancer 2021

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		