PAP-ARMS®

Human PAX1 Gene Methylation Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

Product Name

Human PAX1 Gene Methylation Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Packing Specification

20 Tests/Kit

Intended use

This kit is used to qualitatively detect the methylation of specific sites of PAX1 (Paired boxed gene1) gene in the DNA of cervical exfoliated cell samples (Supplementary Table 1). This kit is only used for the auxiliary diagnosis of patients with atypical squamous cells (ASC) and atypical glandular cells (AGC) as the result of TCT test of cervical exfoliated cells. should not be used as the sole basis for individualized treatment of patients. Clinicians should make a comprehensive judgment on the test results based on factors such as the patient's condition, drug indications, treatment response, and other laboratory test indicators.

In cervical tumor cells, methylation of the CpG island in the promoter region of the PAX1 gene can cause gene silencing or inactivation, suggesting that PAX1 may have a potential tumor suppressor role in the occurrence of cervical cancer. At the same time, abnormally elevated methylation rate of PAX1 is closely related to the progression of cervical precancerous lesions and the occurrence of cervical cancer. When the patient's TCT test results are atypical squamous cells (ASC) and atypical glandular cells (AGC), if the patient's own conditions or personal wishes cannot be further checked and confirmed, the cervical exfoliated cell sample collected during the TCT examination can be directly taken for PAX1 gene methylation detection. When the test results suggest that the PAX1 gene is methylated, it indicates that the patient is at increased risk of disease, and further pathological diagnosis is recommended.

Technological Principles

Unmethylated cytosine (Cytosine, C) can be converted into uracil (Uracil, U) after a series of transformations. After PCR reaction, uracil (U) is finally converted into thymine (Thymine, T), that is, unmethylated cytosine (C) will eventually be converted into thymine (T). The methylated cytosine (C) will not change in the modification reaction due to the protective effect of its methyl group, that is, the methylated cytosine (C) will remain cytosine (C) after conversion.

Based on the above differences, using the genome sequence transformed by the PAX1 gene as a template, ARMS primers were designed for specific methylation sites, and the length of the PCR product was about 100bp. The transformed sequence of the human genome housekeeping gene β-actin was selected as the internal reference template, and the length of the PCR product was about 90bp. This kit realizes the detection of the methylation status of specific sites of the PAX1 gene in the sample DNA on the real-time fluorescence quantitative PCR platform, with high specificity and high sensitivity. When the final result was analyzed, the detection of PAX1 methylation sites was indicated by the FAM signal, and the internal control was indicated by the VIC signal. The presence of methylation in the PAX1 gene was called positive, and the absence of methylation was called negative.

Kit Contents

The kit is pre-packed in eight tubes, and each PCR reaction tube contains specific primers, fluorescent probes, dNTPs, magnesium chloride, potassium chloride and purified water. The kit contains four kinds of reaction solutions, which have been pre-packed in eight tubes. The PAX1-1 reaction solution was dispensed in the No. 1 and No. 5 reaction tubes. No. 2 and No. 6 reaction tubes are filled with PAX1-2 reaction solution. No. 3 and No. 7 reaction tubes are filled with PAX1-3 reaction solution. No. 4 and No. 8 reaction tubes are filled with PAX1-4 reaction solution. No. 1-4 and No. 5-8 reaction tubes of each eight-coupled tube correspond to each other one by one, and one sample is tested respectively.

The PAX1-1 reaction solution, the PAX1-2 reaction solution and the PAX1-3 reaction solution are detection reaction solutions, which are equipped with corresponding PAX1 gene methylation detection reagents and internal control reagents. The detection signal is indicated by the FAM signal, which is used to judge whether there is methylation; the internal control signal is indicated by the VIC signal, which is used to indicate whether the sample is correctly added to the reaction well. The PAX1-4 reaction solution is used as an external control tube for the nucleic acid quality, and contains the external control reagent of the PAX1 gene. The external control signal is indicated by the FAM signal,

which is used to indicate the quality of the sample nucleic acid. Internal and external controls serve as quality controls for reagents, nucleic acid quality and operations.

Table 1 Kit Contents

Component name	Main contents	Volume	Quantity
PAX1 octuple reaction strip	Primer, Probe, Magnesium chloride, dNTPs, Potassium chloride	45 μL	12 Strips
Taq enzyme (PAX1)	Taq DNA polymerase	35 μL	1 Tube
PAX1 positive control	Positive plasmid	100 μL	1 Tube
PAX1 negative control	Purified water	100 μL	1 Tube

Note: Components in kits of different batch numbers cannot be mixed with each other; The reaction solution has been pre sub packed in eight tubes, as shown in Fig. 1, from left to right are tubes 1 to 8.

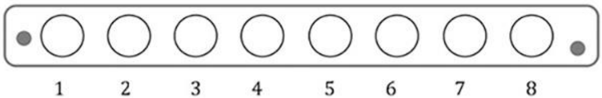


Fig. 1 Schematic diagram of octuple reaction strip

Equipment and Reagents Required

1. It is recommended to use the nucleic acid extraction reagent (Micro DNA) of Xiamen Spacegen Co., Ltd. for nucleic acid extraction (MIN XIA XB No. 20200194);
2. It is suggested to use the genomics DNA bisulfite modification reagent (MIN XIA XB No. 20200141) of Xiamen Spacegen Co., Ltd. for DNA methylation transformation;
3. Purified water without DNase and RNase;
4. No DNase and RNase pipette filter element gun tip.

Transportation, Stability and Storage

1. Storage conditions: the kit shall be stored in a dark place at -20±5°C for 9 months. Storage at -20±5°C after bottle opening does not affect the validity period of the product. The repeated freezing and thawing times of the kit shall not exceed 5 times.
2. Transportation conditions: the kit shall be transported at low temperature, the transportation time shall not exceed one week, and the transportation temperature shall not exceed 25°C.
3. See the label for the production date and validity period.

Applicable instruments

1. ABI7500, ABI 7300Plus.
2. Matters needing attention:
   a) Use the probe mode setting of ABI instrument, reporter dye: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE;
   b) The use of ABI instrument requires manual adjustment of the threshold line, which shall not be less than 20000.

Specimen Material

1. Applicable specimen type: cervical exfoliated cell sample;
2. Samples were collected with commercial cervical brush; When taking samples, pay attention to cleaning the sampling part with sterile normal saline to avoid taking pus as far as possible;
3. If the collected samples cannot be extracted and tested in time, they should be stored at 2 ~ 8°C for no more than 3 days, or at -20±5°C for no more than 30 days. Do not freeze and thaw the samples repeatedly;

4. Genomics DNA was extracted with a commercial extraction kit, and the sample DNA quality and concentration were measured with a micro spectrophotometer. The OD_{260}/OD_{280} value should be in the range of 1.7 ~ 2.2, and the concentration should be greater than 25 ng/μL. The total amount needs to be 0.5 ~ 2 μg. If the DNA quality and concentration do not meet the requirements, the samples shall be taken again or the sample size shall be expanded for DNA extraction again;
5. The extracted DNA is recommended to be transformed immediately or stored at -20±5°C for no more than 12 months and repeated freezing and thawing times no more than 5 times;
6. The modified genome is recommended to be used for detection immediately or stored at -20±5°C for no more than 30 days and repeated freezing and thawing times no more than 3 times.

Experimental Procedure

1. Reagent preparation (reagent preparation area)
   a) According to the number of samples to be tested, take out the PAX1 octuple reaction strip and Taq enzyme (PAX1) in the kit, place them in the ice box and move them to the sample processing area. It is recommended that samples, positive control (PC) and negative control (NTC) be analyzed at the same time in each PCR reaction.
2. Specimen handling (specimen handling area)
   a) The operators use the genomics DNA bisulfite modification reagent of Xiamen Spacegen Co., Ltd. for DNA modification and transformation. Post-transformation genomes were determined according to the concentration using a microspectrophotometer. Take part of the modified samples and dilute them to 20 μL with DNase and RNase-free purified water, with a concentration of 2-5 ng/μL, which is the DNA to be tested;
   b) The operators add 1 μL of Taq enzyme (PAX1) to 20 μL of tested DNA to be tested, PAX1 positive control and negative control respectively, vortex to mix well and centrifuge quickly, which is the amplification template;
   c) The operators gently lift the cover of the PAX1 8-tube reaction strip (placed in the ice box). Add 5 μL of the DNA/PAX1 positive control/negative control amplification template of the above-mentioned tested sample to be tested in turn, and add it to each tube of the PAX1 8-tube reaction strip according to the example in the figure below. Carefully cover the 8-tube reaction strip and move it to the amplification detection area.

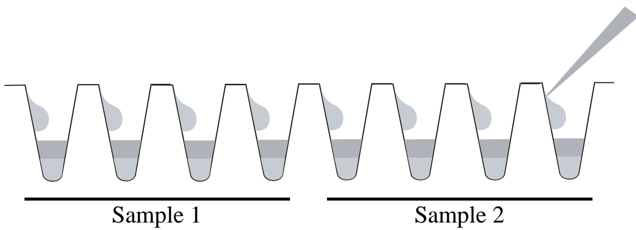


Fig. 2 Schematic diagram of eight pipe reaction strip sampling

3. On machine detection (amplification detection area)
   a) The operators rapidly centrifuge the 8-tube reaction strip for 10 seconds to collect the added amplification template to the bottom of the reaction tube;
   b) The operators put the reaction strip into the real-time PCR instrument, and refer to table 2 for the layout of PCR reaction board;

Table 2 Recommended layout of 96 well PCR reaction plate

Reaction liquid	Pipe No	1	2	3	4	5	6	7	8	9	10	11	12
PAX1-1	**1**	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	PC
PAX1-2	**2**	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	PC
PAX1-3	**3**	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	PC
PAX1-4	**4**	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	PC

Reaction liquid	Pipe No	1	2	3	4	5	6	7	8	9	10	11	12
PAX1-1	**5**	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	NTC
PAX1-2	**6**	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	NTC
PAX1-3	**7**	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	NTC
PAX1-4	**8**	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	NTC

c) The operators open the instrument window and set the amplification program according to the following figure;

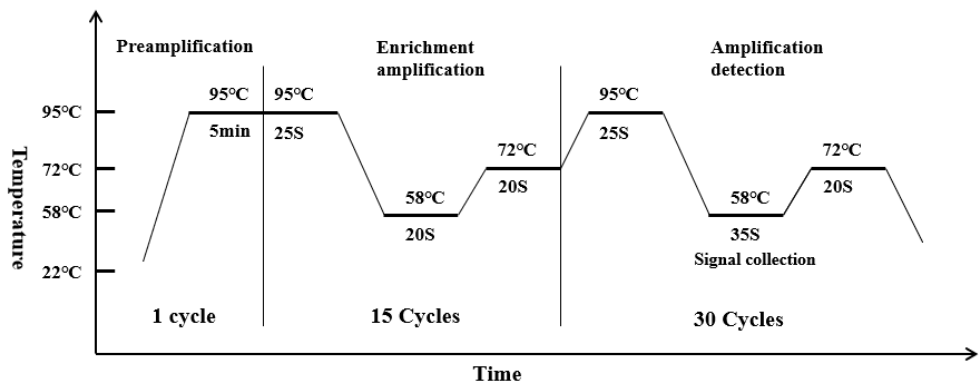


Fig. 3 PCR amplification procedure

d) The operators execute real-time PCR and save the file;

e) After the experiment, isolate the PCR reaction strip and do not open the PCR tube cover to prevent pollution.

Positive judgment value

1. Through clinical experiment analysis, the contents of PAX1-1, PAX1-2 and PAX1-3 were determined by ROC curve method, ΔCt cut off values are 8, 9 and 9.
2. Result judgment
   a) Sample Ct value: A threshold line was delineated using the Ct value of the amplification curve calculated by the instrument software or based on the fluorescence signal of the amplification curve of the positive control FAM channel. The fluorescence threshold should be set at the exponential phase of the PCR amplification curve, that is, the inflection point of the curve. The fluorescence value of the first few cycles of curve amplification does not fluctuate much, and the baseline can be delineated by this or can be delineated according to the software.
   b) Result judgment:
      i. There was no amplification curve for FAM channel of reaction tubes No.1 ~ 3 or No.5 ~ 7 of the sample, which was judged as negative.
      ii. When the FAM channel of reaction tubes No. 1~3 or No. 5~7 has an amplification curve, calculate the ΔCt value of the reaction tube according to the following formula. The results were interpreted according to Table 3, and if at least one of the detection reaction wells was positive, it indicated that the sample had methylation.

Formula: ΔCt value = Detection Ct value - External control Ct value

Where: Detection Ct value: test the FAM signal Ct value of the reaction solution (No. 1 ~ 3 or No. 5 ~ 7 reaction tubes) of the sample to be tested;

External control Ct value: the FAM signal Ct value of the external control reaction solution (tube 4 or 8) of the sample to be tested.

Table 3 Result judgment

Methylation status	PAX1-1 (Pipe No. 1 or 5)	PAX1-2 (Pipe No. 2 or 6)	PAX1-3 (Pipe No.3 or 7)
positive	ΔCt <8	ΔCt <9	ΔCt <9
negative	ΔCt ≥8	ΔCt ≥9	ΔCt ≥9

iii. If the result is positive, there is methylation of PAX1 gene in the sample. If the result is negative, there is no methylation of PAX1 gene in the sample or it is lower than the detection limit of this kit.

Interpretation of Results

1. The FAM signal of negative control (NTC) tube shall have no amplification curve; If the FAM signal rises, the test result is invalid; If the VIC signal rises occasionally, it will not affect the judgment of the detection result.
2. The Ct value of positive control (PC) FAM signal and VIC signal should be < 24, which can fluctuate with the threshold setting of different instruments.
3. The FAM signal of the external control reaction tube (No. 4 or No. 8) of the sample to be tested should have an amplification curve rising, and the Ct value should be between 10 and 17. The VIC signal should have an increase in the amplification curve, and the Ct value should be between 10 and 20. The quality control of this step is qualified and then the next step is analyzed. If the FAM signal Ct value is less than 10, it means that the added DNA concentration is too high and should be diluted before doing. If the FAM signal is negative or the Ct value is greater than 17, it means that the added DNA template contains PCR inhibitors or the DNA concentration is too low, and the loading concentration can be increased or the sample modification can be performed again.

Limitation of the Kit

1. The personalized treatment selection of patients should be comprehensively considered in combination with their symptoms, signs, medical history, other laboratory tests and treatment reactions.
2. The negative result can not completely exclude the existence of no methylation of PAX1 gene specific site, and the negative result can also be caused by excessive degradation of nucleic acid or the concentration of target gene in the amplification reaction system is lower than the detection limit.
3. Unreasonable sample collection, transfer and treatment, or improper test operation and test environment may lead to false negative or false positive results.
4. This kit is only used for qualitative detection of methylation at specific sites of human PAX1 gene.
5. The detection is limited to the sample type and detection system mentioned in the manual (including applicable model, nucleic acid extraction reagent, detection method, etc.).

Physical Performance

1. The kit shall be clean in appearance, clearly marked and free of leakage. After melting, the reagent shall be clear without turbidity and precipitation.
2. Five enterprise accuracy references were tested, and the results should be positive.
3. Test 1 enterprise-specific reference material and 2 non-human genome company-specific reference materials, and the results should be negative. Test 1 enterprise-specific reference product outside the detection range of the ki, the result should be negative.
4. Detect 6 reference materials with the minimum detection limit of the enterprise whose concentration is not higher than 2 ng/μL, and the results should be positive.
5. Two enterprise repetitive reference samples were tested in parallel for 10 times, and the results should be positive.

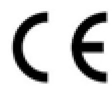
Precautions and Warning

1. Please read this manual carefully before the experiment.
2. Avoid repeatedly freezing and thawing the reagent in the kit.
3. The quality of DNA used for detection is very important. After DNA extraction, quality control shall be carried out to determine the extraction quality, and the next test shall be carried out as soon as possible or stored below -20°C.

4. All reagents in this kit have been specially prepared, and any arbitrary replacement of any reagent may affect the use effect. Components of kits with different batch numbers shall not be mixed with each other.
5. Pay attention to strictly distinguish the use of positive control and reaction reagent to prevent contamination of reagent and false positive results.
6. Pay attention to prevent foreign DNA contamination during the experiment, and ensure that the positive control operation is performed after the sample DNA is added. It is recommended to use separate, dedicated pipettes and filter tips when preparing reaction reagents and adding DNA template. The location where reagent preparation is performed should be isolated from where the template is added.
7. After the experiment, use 10% hypochlorous acid or 75% alcohol or UV light to treat the workbench and pipette.
8. All chemicals are potentially hazardous. This kit can only be used by personnel with a PCR laboratory employment certificate. Before using this kit for the first time, the company's technical support can train operators. When operating, please wear appropriate lab coat and protective gloves.
9. All test samples and positive controls in the kits should be regarded as infectious substances and should be handled with care; the used kits are clinical wastes and should be disposed of properly.

Notes

Symbol	Legend
	Indicates the need for the user to consult the instructions for use.
IVD	Indicates a medical device that is intended to be used as an in vitro diagnostic medical device.
	Indicates the date when the medical device was manufactured.
LOT	Indicates the manufacturer’s batch code so that the batch or lot can be identified.
	Indicates the temperature limits to which the medical device can be safely exposed.
	Indicates the date after which the medical device is not to be used.
	This is the correct upright position of the distribution packages for transport or storage.
	Indicates a medical device that needs to be protected from moisture.
	Indicates a medical device that needs protection from light sources.
	Indicates the medical device manufacturer.
EC REP	Indicates the authorized representative in the European Community/European Union.

	The product meets the basic requirements of European in vitro diagnostic medical devices directive 98/79/EC.

Reference

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2. Nikolaidis C, Nena E, Panagopoulou M, et al. PAX1 methylation as an auxiliary biomarker for cervical cancer screening: A meta-analysis. Cancer Epidemiology, 39(5):682-6, 2015.
3. Huang TH, Lai HC, Liu HW, et al. Quantitative analysis of methylation status of the PAX1 gene for detection of cervical cancer. International Journal of Cancer, 20(4): 513-519, 2010.
4. Lai HC, Lin YW, Huang RL, et al. Quantitative DNA methylation analysis detects cervical intraepithelial neoplasms type 3 and worse. Cancer, 116(18): 4266-4274, 2010.
5. Kan YY, Liou YL, Wang HJ, et al. PAX1 methylation as a potential biomarker for cervical cancer screening. International Journal of Cancer, 24(5): 928-932, 2014.
6. Chao TK, Yu CP, Lai CH, et al. Triage of cervical cytological diagnoses of atypical squamous cells by DNA methylation of PAX1. Diagnostics Cytopathology, 41(1):41-46, 2011.

EC REP

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Attached List 1:

Distribution of test sites of kit

Reaction pore	Methylation site
PAX1-1	Chr20:21686443、Chr20:21686445、Chr20:21686450
PAX1-2	Chr20:21686437
PAX1-3	Chr20:21686325、Chr20:21686333、Chr20:21686376、Chr20:21686378