
Human RASSF1A/SHOX2 Gene Methylation Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

For Research Use Only

Product Name

Human RASSF1A/SHOX2 Gene Methylation Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Packing Specification

20 Tests/Kit

Intended Use

This kit uses multiplex fluorescence PCR amplification technology to qualitatively detect methylation status of RASSF1A and SHOX2 genes in DNA isolated from human plasma. This kit is intended for research use only.

SHOX2 belongs to the SHOX gene family and plays a significant role in the development of bones, heart, and nervous system during embryonic formation. SHOX2 is abnormally expressed in lung cancer, breast cancer, and renal cancer. RASSF1A regulates various biological functions such as gene transcription, signal transduction, cell cycle, and cell apoptosis, and can inhibit tumor formation through multiple pathways. Studies have found that the promoter regions of SHOX2 and RASSF1A are highly methylated in plasma samples from lung cancer patients. By detecting the methylation status of RASSF1A and SHOX2 genes in peripheral blood plasma, the detection rate of early-stage lung cancer can be effectively improved, thereby increasing the survival rate of patients.

Technological Principles

This kit uses bisulfite to modify genomic DNA, converting unmethylated cytosines to uracil, while methylated cytosines remain unchanged. Based on this difference, primers and fluorescent probes are designed for specific methylation sites using the converted sequences of RASSF1A and SHOX2 genes as templates; the converted sequence of the human genomic housekeeping gene β -actin is used as an internal control template, with internal control primers and probes designed accordingly. For product analysis, the use of fluorescently labeled probe real-time tracking analysis makes the detection method automatic. This kit enables the detection of methylation status at specific sites of RASSF1A and SHOX2 genes in DNA on a real-time PCR platform, with high specificity and sensitivity. In the final result analysis, the methylation sites of RASSF1A and SHOX2 genes are indicated by FAM signals, while the internal control gene β -actin is indicated by HEX (VIC) signals.

Kit Contents

This kit contains **RS Taq Polymerase**, **RS Positive Control**, **RASSF1A Reaction Mix** and **SHOX2 Reaction Mix** (Table 1). The reaction solutions include reagents for detecting methylation of RASSF1A and SHOX2, as well as internal control reagents. Methylation signals are indicated by FAM signals, and the internal control is indicated by HEX (VIC) signals.

Table 1. Kit Contents

Content Name	Components	Volume	Quantity
RASSF1A Reaction Mix	Primers, probe, MgCl ₂ , dNTPs	1000 μ L	1 tube
SHOX2 Reaction Mix	Primers, probe, MgCl ₂ , dNTPs	1000 μ L	1 tube
RS Taq Polymerase	Hot Start GU Taq DNA Polymerase	15 μ L	1 tube
RS Positive Control	Positive plasmid DNA, internal control plasmid DNA	100 μ L	1 tube

Note: The contents of different batches cannot be mixed.

Additional required Equipment and Materials

- It is recommended to use the Nucleic Acid Extraction Kit (Plasma DNA) from Xiamen Spacegen Co., Ltd. for nucleic acid extraction. Catalog Number is SPG-HSPD001R.
- It is recommended to use the Genomic DNA Bisulfite Kit from Xiamen Spacegen Co., Ltd. for DNA modification transformation. Catalog Number is SPG-DM001R/002R.

3. Nuclease-Free Water.
4. Aerosol filter pipette tips.

Transportation, Stability and Storage

1. Storage Condition. Store the kit away from light at -15°C to -25°C , valid for 9 months. Once opened, reagents can be stored in their original packaging at -15°C to -25°C until the stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 5 freeze-thaw cycles.
2. Transportation Condition. The kit should be transported at low temperature, with transporting time less than one week and transporting temperature lower than 25°C .
3. Check labels for production date and expiration date of the kit.

Compatible PCR Instruments

ABI7500.

1. For ABI instruments, the probe mode setting as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.

Specimen Material

1. Recommended sample types: peripheral blood plasma.
2. Commercialized kit is recommended to extract DNA from the samples. Assess the quality of sample DNA with an microvolume ultraviolet-visible spectrophotometer, the ratio of $\text{OD}_{260}/\text{OD}_{280}$ should be within the range of 1.7-2.2, the concentration is not less than $25\text{ ng}/\mu\text{L}$. Once the DNA quality or concentration was not in conformity with the above requirements, re-extract DNA with new and/or larger input.
3. Proceed extracted DNA to transforamtion immediately or store the DNA at -15°C to -25°C for no more than 12 months. Freeze-thaw samples no more than 5 times. Proceed transformed DNA to detection immediately or store the DNA at -15°C to -25°C for no more than 1month. Freeze-thaw samples no more than 3 times.

Experimental Procedure

1. Reagent Preparation

Prepare **RASSF1A Reaction Mix**, **SHOX2 Reaction Mix** and **RS Taq Polymerase** from the kit and put them in an ice box. After the reaction mix melts, take out $35\ \mu\text{L}$ of each reaction mix and sequentially add them into the corresponding wells of the 8-tube strip (each 8-tube strip is used to test 4 samples), and add $0.25\ \mu\text{L}$ of **RS Taq Polymerase** to each well. Cover the cap and place 8-tube strips in an ice box to move to the sample processing area. Detection of **RS Positive Control (PC)** and Negative Control (NTC, Nuclease-Free Water) in each reaction/run is recommended.

2. Sample Processing

- (1) Nucleic Acid Extraction Kit (Plasma DNA) is used to extract DNA from peripheral blood plasma samples.
- (2) According to the instructions of the Genomic DNA Bisulfite Kit to modify and transform the tested DNA, the total input of DNA is 300 ng , the elution volume after transformation is $20\ \mu\text{L}$, which is so called tested BisDNA.
- (3) Gently remove the cap of 8-tube strip, sequentially add $5\ \mu\text{L}$ of tested BisDNA, NTC, and PC into tubes of each strip, cover the cap carefully.

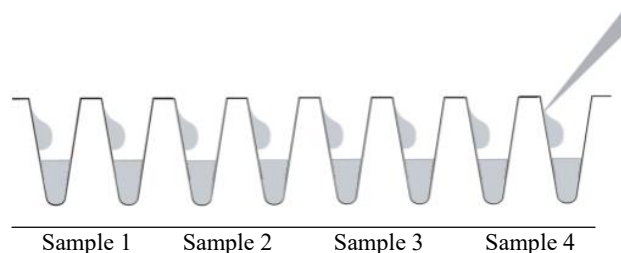


Figure 1. The 8-Tube Strip Sampling Diagram

3. Amplification

- (1) Centrifuge the 8-tube strips for 10 seconds to collect templates.
- (2) Load the 8-tube strips into the real-time PCR instrumen.
- (3) Set and run the amplification program as shown in Figure 2.

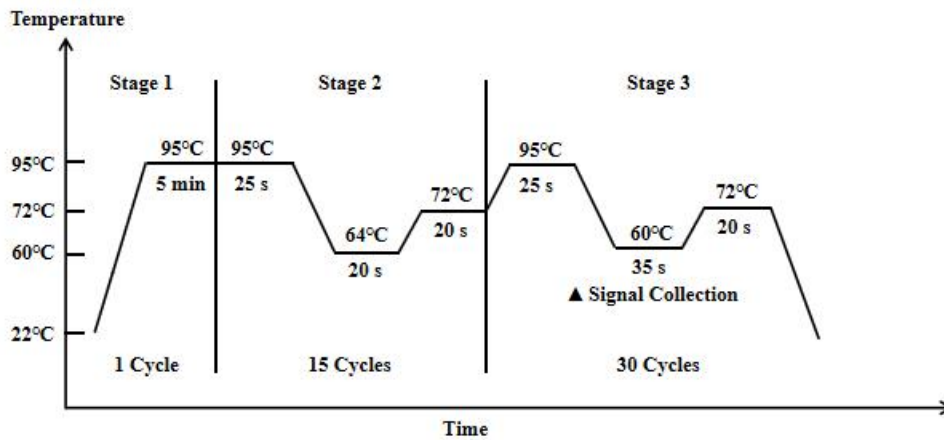


Figure 2. PCR Amplification Procedure

- (4) Handle the strips properly after experiment; do not remove the caps in case contamination.

Data Analysis

1. The positive value of RASSF1A, SHOX2 assay in this kit was determined as 9, 8 respectively with the assist of ROC curve method.
2. Result Judgment

- (1) Ct value: provided by the instrument software or by determining the threshold fluorescence of actual amplification curve.
- (2) Result Judgment (refer to Table 2):
 - a) When there are no FAM signal amplification curve rises, a negative call, or lower than the detection limit of the kit is returned.
 - b) When the FAM signal amplification curve rises, calculate the ΔCt Cut-off value per the equation below. If the derived ΔCt Cut-off value is less than the stated, a positive call is returned for the corresponding gene; otherwise, a negative call or lower than the detection limit of the kit is returned.

$$\text{Equation: } \Delta Ct \text{ Cut-off} = Ct(\text{FAM}) - Ct(\text{HEX/VIC})$$

Table 2. Result Judgment

Methylation		RASSF1A	SHOX2
Positive	Stated ΔCt Cut-off value	$\Delta Ct < 9$	$\Delta Ct < 8$
Negative	Stated ΔCt Cut-off value	$\Delta Ct \geq 9$	$\Delta Ct \geq 8$

- c) A positive call is returned, regardless one or both RASSF1A gene and the SHOX2 gene were positive.
- d) A negative call is returned, if the result of RASSF1A gene and the SHOX2 gene were both negative.

Interpretation of Results

1. NTC: There should be no amplification curves of FAM in NTC reaction tube, or else, call the result invalid. Occasionally, amplification curve of HEX (VIC) generates, which has no influence on result interpretation.
2. PC: There should be amplification curves of FAM and HEX (VIC), with the value of Ct is less than 20, otherwise, the value is invalid and retest is recommended.
3. Internal Control: Amplification curves of HEX (VIC) should generate in every tested sample, and the Ct value should be 12-20, which must be qualified before proceeding to further analysis; If the HEX (VIC) Ct is less than 12, that indicates excessive BisDNA amount,

dilute sample BisDNA for a new detection; If the HEX (VIC) Ct is greater than 20, that indicates insufficient BisDNA amount or that sample BisDNA was contaminated by PCR inhibitor, in this case, it is recommended to increase the input amount and re-transform sample DNA for a new detection.

Limitations of the Kit

1. Negative results could not exclude the existence of RASSF1A/SHOX2 gene methylation; Cases like DNA degradation, or insufficient DNA amount may lead to negative results as well.
2. Situations that may result in false negative or false positive result include but not limit to unreasonable sample collection, transportation, improper experimental operations or environment.
3. The kit is only intended for the qualitative detection of methylation status of specific RASSF1A/SHOX2 gene sites.
4. The kit is only applicable with the stated sample types and detection system, including specified instruments, DNA extraction and transformation kit, and analytical assay.










Performance Characteristics

1. The kit should be of neat appearance, clear labels, and of no leakage. When unfrozen, the reagents shall be clear, without precipitate.
2. The consistency rates of both positive and negative reference materials are 100%.
3. The kit allows the detection of the minimum detection limit reference material which the methylation ratio not higher than 1% and the result should be positive.
4. Repeat the test 10 times for the same precision reference material, the Ct values of both FAM and HEX (VIC) are less than 25, and the coefficient of variation (CV, %) of the Ct value should be less than 10%.

Warnings and Precautions

1. Please read the instruction carefully in prior to experiments.
2. Conduct experiments abided by laboratory regulations to reduce cross-contaminations of products or reagents; divide experiment areas into different function zones if possible.
3. Avoid repetitively freezing and thawing the reagents in the kit. Do not exceed a maximum of 5 freeze-thaw cycles.
4. The results of this kit will be affected by sample source, collection process, quality, transportation conditions, pre-treatment, etc., as well as the quality of the extracted DNA, instrument types, operating environment, and the limitation of current molecular biotechnology. The factors and variables mentioned above would lead to false positive or false negative results. Users must be aware of the potential errors, accuracy and limitations that may exist during the process of detection.
5. The quality of DNA is crucial, and the quality control of DNA should be performed after extraction; proceed to further steps immediately or store properly at -15°C to -25°C.
6. Do not substitute any original reagents contained in the kit. Do not mix reagents with different Lots.
7. Pay special attention to the use of positive control and the use of filter pipette tips is highly recommended to avoid false-positive results caused by contamination of reagents.
8. Be cautious of contamination from external DNA; Ensure to add the DNA template before operating the positive control; Segregate areas for reagent preparation and sample processing; use dedicated pipettes and pipette tips for reagent preparation and template addition, respectively.
9. Clean experiment areas before experiment with 10% hypochlorous acid followed by twice water rinsing; Sterilize the environment and pipettes with 10% hypochlorous acid, 75% ethyl alcohol, or UV radiation after experiment.
10. All the reagents in use have potential hazard. It is recommended wearing proper protective suit and gloves. For first-use of this kit, you may receive training by our technical supports.
11. All samples including positive control in the kit should be considered as potential infectious substances which should be handled carefully.

Symbols

Symbol	Symbol Definition
	Indicates the need for the user to consult the instructions for use.
	Indicates the date when the medical device is manufactured.
	Indicates the manufacturer's batch code so that the batch or lot can be identified.
	Indicates the temperature limitation.
	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 should be kept dry.
	Indicates a medical device that needs protection from light sources.
	Indicates the medical device manufacturer.

References

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2. Schmidt B, Liebenberg V, Dietrich D, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates [J]. *BMC Cancer*, 2010, (10): 600-608.
3. Ren M.P., et al. Methylation analysis of SHOX2 and RASSF1A in bronchoalveolar lavage fluid for early lung cancer diagnosis. *Annals of Diagnostic Pathology* 27(2017): 57-61.
4. Schneider KU, Dietrich D, Fleischhacker M, et al. Correlation of SHOX2 gene amplification and DNA methylation in lung cancer tumors [J]. *BMC Cancer*, 2011, (11): 102-110.
5. Kneip C, Schmidt B, Seegebarth A, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma [J]. *Journal of Thoracic Oncology*, 2011, 6(10): 1732-1638.



Manufacturer: XIAMEN SPACEGEN CO., LTD.
Address: 4th floor, No.2041 Xizhou Road, Tong'an District, Xiamen 361100, P. R. China
Tel: +86 592 7578317 Fax: +86 592 7578319
E-mail: spacegen@ispacegen.com
Website: <http://www.sspacegen.com>