MANUAL

RayBio® Septin9 and NDRG4 gene methylation detection kit

Catalog #: PCR-S9N4

ISO 13485:2016

Introduction

RayBio® Septin9 and NDRG4 gene methylation detection kit is a real-time PCR assay for the detection of abnormal DNA methylation in cell free DNA extracted from human plasma. This kit is based on Taqman™ DNA polymerase amplification and fluorochrome-labeled specific probe reporting. Following bisulfite conversion of unmethylated cytosine nucleotides to uracil, this kit detects methylation of septin9 and NDRG4 genes along with an internal control, actb to monitor sample collection, DNA extraction, and amplification.

Purpose

This kit is used for the qualitative in vitro detection of methylated septin9 and NDRG4 genes in cell free DNA extracted from human plasma.

Kit Components (96 sample kit)

Component	Catalogue #	Specification	Quantity
Reaction Solution	PCR-S9N4-MM	50 µL	2 tubes
Primer and Probe Mix	PCR-S9N4-PP	1.0 mL	2 tubes
Positive Control	PCR-S9N4-POS	150 µL	2 tubes
Negative Control	PCR-S9N4-NEG	150 µL	2 tubes

Note: Do not mix reagents from different lots.

Storage

The kit can be stored at -20°C for a period of 12 months prior to opening. After opening, the reagents are valid for at least 6 months if stored at -20°C.

Required Materials (NOT INCLUDED)

1. Fluorescence PCR instrument capable of reading FAM (494nm maximum absorption, 518nm maximum emission) and JOE (520nm maximum absorption, 545nm maximum emission). *Note: VIC channel can be used in place of JOE on instruments for which JOE has not been calibrated.*
2. Vortex Mixer
3. Microcentrifuge
4. Pipettes
5. Sterile nuclease-free pipette tips (barrier tips recommended) and microfuge tubes
6. Compatible PCR Plate
7. Contact our technical support team for questions about compatibility: techsupport@raybiotech.com

Sample Requirements

1. Sample type: Human plasma. All samples should be regarded as potentially infectious and handled with extreme caution. Measures should be taken to minimize the risk of laboratory transmission based on risk assessment when testing any samples. These precautions, at a minimum, should include proficiency and competency testing, appropriate PPE, avoiding aerosols, and using effective disinfectants (quaternary ammonium compounds and 0.5% bleach).
2. Sample collection: Use K2 EDTA or nucleic acid blood tube to collect 10 mL of blood according to the manufacturer's recommendations. Following collection, K2 EDTA blood collection tubes should be stored at 2-8°C and treated within 24 hours after collection (do not freeze). Free nucleic acid blood collection tubes can be stored at 15-25°C for up to 72 hours before treatment.
3. Sample preparation:
    i. Spin blood collection tube at 1350 ± 150rcf for 12 minutes.
    Note: Ensure that the brake function is turned off to prevent damage to the hemostatic cell layer.
    ii. Aseptically transfer plasma to an appropriately sized centrifuge tube and spin again at 1350 ± 150rcf for 12 minutes.
    iii. Aseptically transfer 3.5 mL of plasma into a new centrifuge tube and mark (at minimum) the sample number.
    iv. Plasma samples can be used immediately or stored at 2 to 8°C for 24 hours, -20°C for one month, or -70°C for six months before being treated per instructions in step 4.
4. Sample processing:
    i. Following plasma collection, cell free DNA should be extracted and then treated with an appropriate bisulfite conversion kit to convert non-methylated cytosine residues to uracil.
    ii. Methods and/or kits used for purification and subsequent bisulfite conversion of cell free DNA should be approved for in vitro diagnostic use in humans.

General Considerations

1. To prevent contamination of PCR reactions, clean and decontaminate all working surfaces, centrifuges, pipets, and other equipment with 10% bleach or DNA Away®, followed by 70% Ethanol before every assay.
2. Conduct sample processing and DNA extraction in a separate area from the PCR assay setup (below termed the "**PCR Assay Setup Area**").
3. Care should be taken to avoid contamination of samples and reactions with DNA amplicons from used PCR plates. It is thus recommended to place used PCR plates in a sealed bag and discard them appropriately. Never open a used PCR plate or place it in the same area as the PCR instrument.
4. To minimize cross-contamination between experimental samples, disposable pipettes and filtered pipette tips are recommended.

Testing Method

1. Reagent preparation
   1.1. Thaw reagents: Remove reaction solution, positive control, and negative control from the kit and fully thaw to room temperature. After thawing, mix gently by pipetting. Briefly centrifuge to collect contents at bottom of the vial.
   1.2. Calculate number of reactions needed: The number of reactions to be prepared per PCR run is calculated as: # Reactions = n + 1 positive control + 1 negative control + 2, where "n" is the number of samples (plus any replicates). *Note*: 2 additional reaction volumes are factored into the above formula to account for pipetting error.
   Example: For 35 samples with no replicates, total reactions = 35 + 1 + 1 + 2 = 39.
   1.3. Prepare PCR Master Mix: For each reaction, combine 19 µL primer and probe mix (PCR-S9N4-PP) with 1 µL reaction solution (PCR-S9N4-MM) and mix well. To calculate the total volume necessary for each run, multiply the volumes of each component by the number of reactions calculated in step 1.2.
2. Sample Loading (PCR Assay Setup Area)
   2.1. Add 20 µl of prepared PCR Reaction Mix from step 1.3 to each well of a PCR reaction plate.
   2.2. Add 30 µl of treated cell free DNA, positive control, or negative control to appropriate wells and mix by pipetting up and down at least 5 times.
   2.3. Seal the plate or tubes tightly.
   2.4. Centrifuge the plate or tubes for 30 seconds at low speed. *Note*: The sealed PCR reaction tubes can be stored at 2-8°C for up to 4 hours before the "PCR Amplification" step 3 below.
3. PCR Amplification (PCR Assay Setup Area)
   3.1. Sample setup: Set the sample number, negative control, and positive controls according to your 96-

well plate setup.

3.2. Fluorescence channel selection: Select FAM and set target name to “Septin9/NDRG4.” Select JOE and set the target name to “IAC.” This channel will detect the actb internal amplification control. In the “reference fluorescence dye” (passive reference), set to “None”.

3.3. Set reaction conditions according to Table 1. Set the reaction volume to 50 µL.

Table 1: PCR Program

Step		Temperature (°C)	Time	Temperature Ramp Rate	Number of Cycles
Stage 1	Initial Denaturation	95	5 min	100%	1
Stage 2	Denature	95	5 s	100%	45
	Annealing, extension, and fluorescence detection	55	35 s	100%	

3.4. Save the file and run the program. Sample images of PCR amplification are shown in Figure 1.

4. Results and Analysis

4.1. Set baseline: The start and end cycle of the baseline region is automatically determined by the instrument but can be manually adjusted by the user for better baseline control. If manually setting the baseline, the start cycle can be set between 2 and 8, and the end cycle can be set between 10 and 20.

4.2. Set FAM threshold: The fluorescence threshold for the FAM channel is manually set just above the highest point of the amplification curve of the negative control. When set correctly, the Cq value of the negative control will be undefined, whereas that of the positive control will be less than or equal to 41. See Figure 1 for an example.

4.3. Set JOE threshold: The fluorescence threshold for the JOE channel is automatically determined by the instrument software. See Figure 2 for an example.

4.4. Quality control: The positive and negative controls are considered valid if they meet the criteria listed in Table 2. The PCR reaction is invalid if 1) the positive control does not have logarithmic growth or the FAM channel Cq > 41, 2) the negative control has a FAM channel Cq ≤ 45, or 3) either the negative or positive control has a JOE channel Cq > 32. If the reaction is invalid, the measurement of all samples in the experiment should be discarded, and the assay repeated.

Table 2: Validation of PCR reaction with quality control samples

Quality control sample	Fam channel	JOE channel
Positive control sample	Cq ≤ 41	Cq ≤ 32
Negative control sample	No logarithmic growth period amplification curve or Cq = 45	Cq ≤ 32

4.5. Interpretation of test results: The PCR reaction sample results are explained according to Table 3.

Table 3: Interpretation of test results

PCR reaction results	Fam channel	JOE channel
Positive	Cq < 45	Cq ≤ 32
Negative	No logarithmic growth period amplification curve or Cq= 45	Cq ≤ 32
Invalid	Any results	Cq > 32

Positive Result: the sample contains the target genes.

Negative Result: the sample does not contain the target genes.

Invalid Result: the sample should be rerun with fresh samples and controls.

Product Performance Index

1. Limit of Detection**:** The LOD of the assay is 6.25 pg/mL.
2. Repeatability**:** Precision testing showed that the coefficient of variation (CV) of the precision Cq values within and between lots is less than 5%.
3. Accuracy**:** Coincidence rate of the negative and positive reference standards was 100%.
4. Clinical sensitivity and specificity**:** Clinical screening consisted of 150 samples from donors with minimal/no risk of disease, 100 from patients diagnosed with colorectal cancer, and 100 from patients determined not to have colorectal cancer. ***Note***: Diagnoses of the 100 colorectal cancer donors and 100 "healthy" donors were made by an independent third party according to colonoscopy results and/or histological examination. Of the 150 screening samples, all but 1 were negative, a false positive rate of 0.7%. Among 100 normal control subjects, 98 were negative, and the clinical specificity was 98%. Among the 100 samples diagnosed with colorectal cancer, 85 samples were positive, with a clinical sensitivity of 85%. Among the 56 patients with early colorectal cancer, 45 (80%) were detected positive by the kit, including 20 cases in stage I and 25 cases in stage II.
5. Interference: Unmethylated DNA (100 ng/ml), bilirubin (0.20 mg/ml), hemoglobin (1 mg/ml), triglyceride (12 mg/ml), protein (serum albumin) (120 mg/ml), red blood cells (0.4% v/v), k2edta (20 mg/ml), cholesterol (5 mg/ml), uric acid (0.235 mg/ml) and glucose (10 mg/ml) were not observed to interfere with the experiment in the experimental control and non-reactive or reactive test samples.

Limitations

1. This kit is only to be used for in vitro diagnosis.
2. This kit was validated using nucleic acid purification reagents from RayBiotech and was not validated with reagents from other manufacturers. Independent assay will need to be conducted if using third-party cell free DNA extraction and/or bisulfite conversion kits.
3. Assay performance has been validated with blood samples collected in K2 EDTA and free nucleic acid blood tubes. No testing was completed for other blood sample collection modalities.
4. Users of this product should be trained to conduct real time PCR diagnostic tests.
5. Since detection of colorectal cancer relies on the amount of tumor DNA in the sample, it may be influenced by the sample collection process, the mode of sample storage, or individual patient factors (e.g., age, other diseases), and the grade of the tumor.
6. A positive result should not be interpreted as proof of colorectal cancer. Patients who test positive with the septin9 and NDRG4 methylation test should undergo subsequent screening to confirm a diagnosis of colorectal cancer (e.g., colonoscopy, sigmoidoscopy, etc.).
7. False positive test results have been observed in pregnant women and patients with the following clinically diagnosed diseases: chronic gastritis, esophagitis, non-rheumatoid arthritis, lung, breast, and prostate cancer.
8. The kit assay results should be evaluated in conjunction with other clinical indicators.