Ring-Cap ®

Colorectal Cancer Related Gene Mutation Detection Kit

High Throughput Sequencing

Instruction for Use

Product Name

Colorectal Cancer Related Gene Mutation Detection Kit (High Throughput Sequencing)

Packing Specification

16 Tests/kit, 32 Tests/kit

Intended Use

This kit is used to qualitatively detect 5 hotspot mutation genes (KRAS, NRAS, BRAF, PIK3CA, TP53), 2 chemotherapy-related genes (UGT1A1, DPYD), and ERBB2 copy number variations in formalin-fixed paraffin-embedded tissue samples from colorectal cancer patients, and the detection of 34 microsatellite loci to analyze the microsatellite instability status of patients (Attached table 1). The test results are only to evaluate the molecular characteristics of colorectal cancer patients to provide clinical reference, and should not be used as the sole basis for individualized treatment of colorectal cancer patients. Clinicians should consider the patient's condition, drug indications, treatment response and other laboratory test indicators. and other factors to make a comprehensive judgment on the test results.

Technological Principles

High-throughput sequencing (High-Throughput Sequencing), also known as Next Generation Sequencing (NGS), can achieve parallel sequencing of hundreds of thousands or even millions of target nucleic acid molecules at a time, with high output and high resolution. The characteristic of a high degree of accuracy not only provides rich sequence variation information, but also greatly reduces the cost and time-consuming of sequencing, and plays a significant role in multi-path and multi-target cancer research.

Using DNA extracted from FFPE samples, the target gene was amplified and enriched by designing multiple PCR primers, and then the sample library was obtained by PCR amplification by primers with index sequences (Index) and RingCap-Taq polymerase. The sequencing data of the library is obtained by high-throughput sequencing, and the final gene variation information and microsatellite instability status can be obtained after being analyzed by the bioinformatics analysis software.

Kit Contents

Table 1. Kit Contents

Number	Component Name	Color	16 Tests / kit			32 Tests / kit			Notes
			Volume	Tube Number	8-tube strip	Volume	Tube Number	8-tube strip	
1	CRC-1 reaction strip	Blue	20 μL	16 Tubes	2 Strips	20 μL	32 Tubes	4 Strips	Each tube contains same reagent.
2	CRC-2 reaction strip	Pink	20 μL	16 Tubes	2 Strips	20 μL	32 Tubes	4 Strips	Each tube contains same reagent.
3	Index 1-8 reaction strip	Purple	20 μL	8 Tubes	1 Strip	20 μL	8 Tubes	1 Strip	Each tube represents an index.
4	Index 9-16 reaction strip	Green	20 μL	8 Tubes	1 Strip	20 μL	8 Tubes	1 Strip	Each tube represents an index.
5	Index 17-24 reaction strip	White	——	——	——	20 μL	8 Tubes	1 Strip	Each tube represents an index.
6	Index 25-32 reaction strip	Yellow	——	——	——	20 μL	8 Tubes	1 Strip	Each tube represents an index.
7	RingCap-Taq (1#)	——	20 μL	1 Tube	——	20 μL	2 Tubes	——	——
8	CRC negative control	——	50 μL	1 Tube	——	50 μL	1 Tube	——	——
9	CRC positive control	——	50 μL	1 Tube	——	50 μL	1 Tube	——	——

Note: In the Index reaction system, different serial numbers contain 32 different IlIDx recognition sequences respectively, and the specific sequence information is shown in Attached Table 3. The reaction solution has been pre-loaded in eight strips, and the eight tube caps are inclined to the left in the positive direction. From left to right, the tubes are Index 1, 2, 3, 4, 5, 6, 7, 8, as shown in Figure 1.

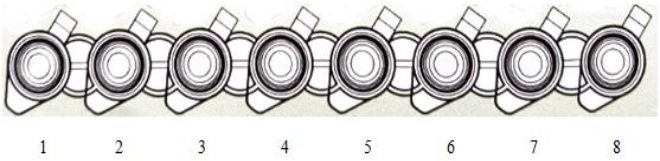

Figure 1. Schematic diagram of eight tubes

Note: Components in kits of different batch numbers cannot be mixed with each other.

Equipment and Reagents Required

1. Nucleic acid extraction kit: It is recommended to use nucleic acid extraction reagents from Xiamen Spacegen Co., Ltd;
2. Fluorometer: It is recommended to use the Qubit™ 4 Fluorometer from Thermo Fisher Scientific, CAT. No:Q33238 and Quantus™ Fluorometer from Promega, CAT. No E6150;
3. Quantification kit of nucleic acids: Promega QuantiFluor®ds DNA System, Cat. No. E2670, Qubit® dsDNA HS Assay Kit, Cat. No. Q32851/Q32854;
4. Sequencing reagents: Select the corresponding sequencing reagents according to the gene sequencer used ;
5. Illumina PhiX Control v3 (Illumina), Cat. No.: FC-110-3002;
6. Magnetic beads: Use Magnetic beads kit from Beckman Coulter, product number A63880/A63881/A63882; SGpure beads from Xiamen Spacegen Co., Ltd, Cat. No. SPG-PB001;
7. Magnetic rack;
8. Nuclease-free water;
9. Anhydrous ethanol (analytical grade);
10. Nuclease-free pipettes and tips;
11. TE (pH 8.0) buffer;

Storage and Stability

1. Storage Condition. Store the kit away from light at -20±5 °C, valid for 9 months. Store the kit upright. Do not use the reagents after 5 freeze-thaw cycles. Once opened, the kit is stable at -20±5 °C until the stated expiration date.
2. Transportation Condition. The kit should be transported in foam cases with ice bags, 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.

Applicable Instruments

1. PCR system/ thermal cycler: ABI9700, ABI 2720, ABI Veriti, ABI Mini Amp.
2. Sequencing instruments: Illumina sequencing platforms.

Specimen Material

The quality of the DNA to be tested is critical. During clinical operations, please collect samples according to the following recommended sample types, and then perform DNA extraction:

1. Recommended tumor sample type: FFPE;
2. FFPE pathological tissue or sections: It should be determined that it contains at least 20% tumor lesions. It is recommended to select samples with a storage period of less than 2 years, and use no less than 8 slices of 5 μm or no less than 5 slices of 10 μm section for DNA extraction;
3. Use the recommended nucleic acid extraction kit for DNA extraction, and use a UV spectrophotometer to judge the quality of the extracted DNA. Its OD_{260}/OD_{280} should be in the range of 1.8 - 2.2; Use a fluorometer to measure the effective DNA concentration, and the DNA concentration should be > 5 ng/μL, the total amount of DNA should be more than 50 ng; If the DNA concentration does not meet the requirements, resampling or expanding the sample volume before DNA extraction; The extracted DNA should be constructed immediately or stored below - 20±5℃ for no more than 12 months.

Experimental Procedure

Note: It is recommended to construct the library of the sample to be tested, the CRC positive control(PC) and the CRC negative control(NC) at the same time.

I. Library Enrichment

1. Preparation of enriched PCR reaction reagents: Remove the " CRC-1 reaction strip (blue)" and " CRC-2 reaction strip (pink)" and " RingCap-Taq (1#)" from the refrigerator;
2. Thawed " CRC-1 reaction strip (blue)" and " CRC-2 reaction strip (pink)" at room temperature, followed by rapid centrifugation; centrifuge "RingCap-Taq (1#)", then place it on the ice box for use;
3. DNA preparation of the sample: Dilute the DNA extracted from the FFPE tissue to 5 ng/μL, the volume is ≥ 10 μL;
4. Add 0.25 μL of "RingCap-Taq (1#)" to the " CRC-1 reaction strip (blue)" and " CRC-2 reaction strip (pink)" prepared in the previous step, followed by rapid centrifugation;
5. Add 5 μL of the above samples along the tube wall to the " CRC-1 reaction strip (blue)" and " CRC-2 reaction strip (pink)", cover the lids for centrifuge, and avoid the bubbles;
6. Put the reaction tube into the PCR machine, and set the amplification program according to Table 2.

Table 2 PCR amplification procedure of library enrichment reaction

Stage	Temperature	Time	Number of Cycles
Pre denaturation	98 ℃	2 minutes	1
Denaturation	98 ℃	15 seconds	15
Annealing	65 ℃	4 minutes	
Preservation	4 ℃	∞	1

Note: If the follow-up operation is not carried out immediately, the reaction products should be stored at low temperature; Store at 2 - 8℃ within 4 hours; Store at - 20±5℃ within 24 hours; It is recommended that the storage time should not exceed 24 hours.

II. Purification of Enriching Products

Note: Bring magnetic bead to room temperature and vortex thoroughly to disperse magnetic bead before use; prepare fresh 70% ethanol with nuclease-free water

1. Mix the reaction products of the CRC-1 reaction strip and the CRC-2 reaction strip then transfer to a new 1.5 mL centrifuge tube, add 50 μL of magnetic beads and mix by pipetting;
2. Incubate at room temperature for 5 minutes;
3. Incubate on a magnetic rack for 2 minutes until the solution is clear, carefully aspirate and discard the supernatant, do not disturb the magnetic beads; Note: Do not discard the magnetic beads that contain enriched products;
4. Add 150 μL of the prepared 70% ethanol solution, place it on a magnetic rack, turn the centrifuge tube clockwise / counterclockwise 5 times, then place it on the magnetic rack and incubate for 2 minutes until the solution is clear, discard the supernatant;
5. Repeat step 4 above;
6. Ensure that the ethanol solution in the centrifuge tube has been completely discarded. Place the centrifuge tube on the magnetic rack, dry it with air at room temperature for 5 minutes. Pay attention to avoid excessive drying;
7. Remove the centrifuge tube from the magnetic rack, add 35 μL TE(pH8.0) buffer to fully infiltrate the magnetic beads, resuspend the magnetic beads, and incubate at room temperature for 5 minutes;
8. Put the centrifuge tube on the magnetic rack for 2 minutes until the solution is clear, transfer the supernatant to a new 1.5 mL centrifuge tube, which is the purified product, store at - 20±5°C or proceed to the next reaction immediately.

III. Library Construction

1. Preparation of Index reaction reagents: Thaw corresponding number of "Index reaction tubes" at room temperature, followed by centrifuging rapidly for use, place the "RingCap-Taq (1#)" on ice box for use after centrifuged;
2. Add 0.25 μL RingCap-Taq (1#) to 5 μL of the purified product, vortex to mix and centrifuge rapidly;
3. Add 5 μL of the above template along the tube wall to the corresponding index, then cover the lids;
4. Centrifuge the Index, avoid bubbles;
5. Put the Index reaction tube into the PCR machine, and set the amplification program according to Table 3.

Table 3 Library Preparation Reaction PCR Amplification Procedure

Stage	Temperature	Time	Number of Cycles
Pre denaturation	98 ℃	2 minutes	1
Denaturation	98 ℃	15 seconds	25
Annealing	65 ℃	4 minutes	
Preservation	4 ℃	∞	1

Note: If the follow-up operation is not carried out immediately, the reaction products should be stored at low temperature; Store at 2 - 8℃ within 4 hours; Store at - 20±5℃ within 24 hours; It is recommended that the storage time should not exceed 24 hours.

IV. Library Purification

Note: Bring magnetic bead to room temperature and vortex thoroughly to disperse magnetic bead before use; prepare fresh 70% ethanol with nuclease-free water.

1. Completely transfer the Index reaction product to a new 1.5 mL centrifuge tube, add 25 μL of magnetic beads and mix by pipetting;
2. Incubate at room temperature for 5 minutes;
3. Incubate on a magnetic rack for 2 minutes until the solution is clear, carefully aspirate and discard the supernatant, do not disturb the magnetic beads; Note: Do not discard the magnetic beads that contain enriched products.
4. Add 150 μL of the prepared 70% ethanol solution, place it on a magnetic rack, turn the centrifuge tube clockwise / counterclockwise 5 times, place it on the magnetic rack and incubate for 2 minutes until the solution is clear, discard the supernatant;
5. Repeat step 4 above;
6. Ensure that the ethanol solution in the centrifuge tube has been completely discarded. Place the centrifuge tube on the magnetic rack, dry it with air at room temperature for 5 minutes. Avoid excessive drying;
7. Remove the centrifuge tube from the magnetic rack, add 35 μL TE(pH8.0) buffer to fully infiltrate the magnetic beads, resuspend the magnetic beads, and incubate at room temperature for 5 minutes;
8. Place the centrifuge tube on the magnetic rack for 2 minutes until the solution is clear, transfer the supernatant to a new 1.5 mL centrifuge tube, which is the sample library, and store at - 20±5°C or proceed to the next steps.

V. Library Quantification and Dilution

1. Library quality control: Use a capillary electrophoresis instrument for library fragment quality control. The negative control, positive control and the main fragments of the tested sample library should be distributed in 250 ~ 350 bp; Use a fluorimeter for library concentration quality control, when the concentration of the fluorimeter is ≥ 1 ng/μL meets the requirements;
2. According to the library concentration measured by the fluorometer, use the following formula to convert the molar concentration of the library, where the DNA length is calculated as 300 bp;

$$\text{Library concentration: nM} = \frac{\text{DNA concentration (ng/μL)} \times 10^6}{\text{DNA length (bp)} \times 650}$$

3. According to the converted molar concentration of the library, use purified water without DNase and RNase to dilute the library to 4 nM;
4. The recommended amount of sequencing throughput per sample is not less than 0.4Gb and concentration of PhiX Control V3 is 5%-15%;
5. The undiluted library can be stored at - 20±5℃ for 7 days; The mixed diluted library is recommended to be used immediately.

VI. Sequencing

Library sequencing was carried out according to the procedure of instrument and matching reagent.

VII. Bioinformatics Analysis

1. Use the Illumina Sequencing Analysis Viewer v1.9.1 software to analyze the quality of the sequencing data, and the Q30 quality value of the data is required to be greater than 75%;
2. Transfer the FastQ files obtained by sequencing to the analysis server, and perform data quality control, sequence alignment,

mutation analysis, mutation annotation and unstable state analysis of microsatellite sites through the clinical high-throughput sequencing data analysis system (hereinafter referred to as the analysis system) of XIAMEN SPACEGEN CO., LTD.;

3. Data filtering: Use the quality control module of the analysis system (based on Trimmomatic v0.36) to remove primer sequences and low-quality bases in the original data;
4. Sequence alignment: Use the sequence alignment module of the analysis system (based on BWA v0.7.17 and SAMtools v1.9) to align the filtered Fastq files to the human reference genome (hg19 version), respectively, to generate BAM files and BAI files;
5. Mutation analysis: Use the mutation detection module of the analysis system (based on Pisces v5.2.9) to detect point mutations and indel mutations in the target region "SG_CRC_target.region_V1.0.bed";
6. Mutation annotation: Annotate identified point mutations and indel mutations according to the AMP-ASCO-CAP 2017 guidelines using the analysis system's variant annotation module (based on ANNOVAR v20180426);
7. Microsatellite instability analysis: Each microsatellite was assessed by comparing the read length distribution between tumor and normal samples (baseline) by chi-square test using the MSI analysis module of the analysis system (based on SG-MSI v1.2) The site is unstable, and the MSI-Score of the sample is obtained by statistical analysis.

Data Analysis

1. View and download the analysis results on the results page of the analysis system.
2. Data quality control standards: It is required that the on target ratio (On target Ration) of sample sequencing data should be greater than 80%, the effective average sequencing depth (Mean Depth) should be greater than 2000 ×, and the amplification uniformity (Uniformity) should be greater than 75%.
3. Positive judgment criteria: In the system variation analysis results, when the effective depth of sequencing is not less than 100 ×, the mutation frequency is not less than 5%, and it is judged as positive, otherwise it is negative or lower than the detection limit of the kit.
4. Analysis of the unstable state of microsatellites, according to MSI-Score to judge the unstable state of microsatellites:
   a) Microsatellite stable (MSS): MSI-Score < 0.3;
   b) Microsatellite instability (High-frequency MSI, MSI-H): MSI-Score ≥ 0.3.

Interpretation of Results

1. The sample DNA library should have strong bands around 250 ~ 350 bp after the capillary electrophoresis test. Through high-throughput sequencing, the on target ratio > 80%, uniformity should be > 75%, and the effective average sequencing depth should be > 2000 ×, otherwise the results of the sample DNA is invalid;
2. The library fragment of the negative control (NC) is about 250 ~ 350 bp, the system variation analysis result should be negative, and the microsatellite is stable, then the negative control library is qualified for quality control. If relevant mutations or microsatellite instability are detected, it indicates that there may be a source of DNA contamination in the environment;
3. The library fragment of the positive control (PC) is about 250 ~ 350 bp, the test results should be consistent with the information of the positive control (Annex table2), then the quality control of the positive control library is qualified. If the result does not match the positive control information, the test result is invalid.

Limitation of the Kit

1. The detection ability of DNA extracted from paraffin tissue samples beyond the detection site range of this kit and stored for a long time is not carried out in accordance with the instructions.
2. Negative results cannot completely rule out the presence of mutations. Few tumor cells in the sample, excessive degradation or the concentration of mutated DNA in the library below the detection limit can also cause negative results.
3. Unreasonable sample collection, transportation and processing, as well as improper experimental operation and experimental environment may lead to false negative or false positive results.
4. Tumor tissue (cells) may have great heterogeneity, and different test results may be obtained by sampling from different tissue parts.

Product Performance

1. The reagent has melted, it should be clear, with no turbidity and no precipitation.

2. 12 positive reference products of the enterprise were tested, and the compliance rate of the positive reference products was 100%.
3. Detect 8 negative reference products with negative mutations and stable microsatellites within the detection range, and the coincidence rate of the negative reference products is 100%.
4. This kit can detect gene mutations as low as 5% in a 25 ng DNA sample.
5. The detection limit of microsatellite instability is 5% of tumor cell DNA content.
6. 2 copies of the enterprise's positive repeatable reference products were tested, each sample was tested 10 times, and the test results were all of the corresponding mutation type or microsatellite instability; The results were all mutation negative and microsatellite stable.
7. The residual hemoglobin, paraffin, formalin and ethanol in the sample nucleic acid will not affect the product if the concentration is not higher than 2 g/L, 1%V/V, 0.005%V/V and 1%V/V.

Precautions and Warnings

1. Please read this manual carefully before experimenting.
2. Follow laboratory management practices to minimize cross-contamination of products and reagents; If conditions permit, the area or room for preparing PCR reactions and subsequent operation areas should be zoned.
3. Before the experiment starts, use 10% hypochlorous acid to clean the experimental table, and then wash it twice with water; After the experiment, use 10% hypochlorous acid or 75% alcohol or UV light to treat the table and pipette.
4. Try to avoid using the peripheral reaction wells of the PCR instrument, and try to reserve wells or columns between sample wells to avoid cross-contamination between samples.
5. The results of this kit may be affected by factors such as sample source, sample collection process, sample quality, sample transportation conditions, sample pretreatment, etc., as well as DNA extraction quality, high-throughput sequencer model, operating environment, and current molecular biology limitations, such as technical limitations, may result in false positive or false negative test results. Users must be aware of potential errors and limitations of accuracy that may exist in the detection process.
6. Avoid unnecessary freezing and thawing of the reagents in the kit, and the number of freezing and thawing is no more than 5 times.
7. The quality of the DNA used in the test is very important. After the extraction is completed, the next step should be carried out as soon as possible or stored at the recommended temperature.
8. All the reagents in this kit have been specially formulated. Any replacement of any of the reagents may affect the use effect. The components of the kits of different batches cannot be mixed with each other.
9. Strict attention to operation, it is recommended to use pipette tips with filter cartridges to prevent reagent contamination and cause false positive results.
10. Pay attention to prevent the contamination of reagents by exogenous nucleic acid. It is recommended to use separate, dedicated pipettes and filter tips when preparing reaction reagents and adding DNA template. The site where the reagents are prepared should be isolated from the site where the template is added.
11. All chemicals are potentially hazardous. Only those who hold a PCR laboratory work permit can use this kit. Before using this kit for the first time, the operator should be trained. When operating, please wear appropriate laboratory coats, gloves and other protective measures. Used kits are clinical waste and should be disposed of properly.
12. 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.

	Indicates the date when the medical device was manufactured.
	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.
	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.

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EC REP

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Attached table 1

Detection Range of this Kit

Gene/ Item	Detection Range
KRAS	Exon 2,3,4
NRAS	Exon 2,3,4
BRAF	V600E
PIK3CA	hot spot
MSI	34 single nucleotide sites
UGT1A1	UGT1A1 * 28 / * 6 (rs3064744, rs4148323)
DYPD	Rs3918290, rs55886062, rs67376798, rs75017182, rs1801265, rs1801159, rs1801160
TP53	CDS
ERBB2	CNV
MSI	34 microsatellite loci

Attached table 2

Positive Control Information for this Kit

System Variation Information				
Gene Name	**Base Mutation**	**Amino Acid Mutant Form**	**Cosmic ID**	**Mutant Type**
KRAS	c.35G>A	p.G12D	521	point mutation
BRAF	c.1799T>A	p.V600E	476	point mutation
Microsatellite instability states: MSI-H				

Attached table 3

32 Different IllDx Recognition Sequence Information on the Illumina Platform

8-tube strip color	Index number	i7 serial	i5 series
purple	Index_001	TAAGGCGA	CTCTCTAT
	Index_002	CGTACTAG	TATCCTCT
	Index_003	AGGCAGAA	GTAAGGAG
	Index_004	TCCTGAGC	ACTGCATA
	Index_005	GGACTCCT	AAGGAGTA
	Index_006	TAGGCATG	CTAAGCCT
	Index_007	CTCTCTAC	CGTCTAAT
	Index_008	CGAGGCTG	TCTCTCCG
green	Index_009	TAAGGCGA	TATCCTCT
	Index_010	CGTACTAG	GTAAGGAG
	Index_011	AGGCAGAA	ACTGCATA
	Index_012	TCCTGAGC	AAGGAGTA
	Index_013	GGACTCCT	CTAAGCCT
	Index_014	TAGGCATG	CGTCTAAT
	Index_015	CTCTCTAC	TCTCTCCG
	Index_016	CGAGGCTG	CTCTCTAT

8-tube strip color	Index number	i7 serial	i5 series
white	Index_017	TAAGGCGA	GTAAGGAG
	Index_018	CGTACTAG	ACTGCATA
	Index_019	AGGCAGAA	AAGGAGTA
	Index_020	TCCTGAGC	CTAAGCCT
	Index_021	GGACTCCT	CGTCTAAT
	Index_022	TAGGCATG	TCTCTCCG
	Index_023	CTCTCTAC	CTCTCTAT
	Index_024	CGAGGCTG	TATCCTCT
yellow	Index_025	TAAGGCGA	ACTGCATA
	Index_026	CGTACTAG	AAGGAGTA
	Index_027	AGGCAGAA	CTAAGCCT
	Index_028	TCCTGAGC	CGTCTAAT
	Index_029	GGACTCCT	TCTCTCCG
	Index_030	TAGGCATG	CTCTCTAT
	Index_031	CTCTCTAC	TATCCTCT
	Index_032	CGAGGCTG	GTAAGGAG