CE

RingCap®

Human Endometrial Cancer Molecular Classification Detection Kit

High-Throughput Sequencing

Instruction for Use

Product Name

Human Endometrial Cancer Molecular Classification Detection Kit (High-Throughput Sequencing)

Packing Specification

16 Tests/Kit, 32 Tests/Kit

Intended Use

This kit is used to qualitatively detect the mutation status of 5 MMR related genes (MSH2, PMS2, MLH1, MSH6, EPCAM), POLE, TP53, PTEN, PIK3CA, KRAS and CTNNB1genes in FFPE samples of endometrial cancer patients, and detect the HER2 gene to analyze the copy number variations status of patients, and detect 34 microsatellite loci to analyze the micro satellite instability status of patients. The test results are only for the evaluation of molecular characteristics of endometrial cancer patients to provide clinical reference, and should not be used as the sole basis for individualized treatment of endometrial cancer. 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.

The endometrial cancer is one of the most common female reproductive system tumors, with nearly 200,000 new cases each year, is the third common gynecological malignant tumor leading to death. The traditional classification of endometrial cancer cannot fully reflect tumor heterogeneity. Therefore, there are certain limitations in predicting the prognosis of patients and the effect of treatment response. In 2013, the Cancer Genome Atlas (TCGA) of the United States identified four new types of endometrial cancer by integrating genomics features: POLE ultra-mutated (POLEmut), micro satellite instability hyper mutated (MSI-H), copy number low (CN-L), copy number high (CN-H).

POLE ultra-mutated (POLEmut): POLE is a catalytic sub unit of DNA polymerase, which is involved in DNA replication and repair. Mutations in the POLE exonuclease domain is seen in 7% of endometrial cancers. This type is characterized by: POLE exonuclease domain (exons 9 - 14) mutations, and the prognosis is better than other types.

Micro satellite instability hyper mutation type (MSI-H): MSI is more distinct in endometrial carcinoma tissues and usually predicts a better clinical stage. MSI-H endometrial carcinoma is characterized by MLH1 promoter hypermethylation or defective mutations in DNA mismatch repair (MMR) related genes which will lead to micro satellite instability (MSI).

Copy number low (CN-L): Low copy number endometrial cancer includes endometriosis cancer with grades 1 to 2 and stable micro satellites. The low gene copy number changes are usually without POLE and TP53 mutations. It is also called non-specific molecular signature type (NSMP).

Copy number high (CN-H): Including all serous carcinomas and 1/4 histologist grade 3 endometriosis carcinomas. Its molecular characteristics include: More changes in gene copy number, most of the cases have TP53 mutation (90%), also known as p53 mutation, and the prognosis is worse than other types.

Technological Principles

High-Throughput Sequencing, also known as Next Generation Sequencing (NGS), can be divided into semiconductor sequencing, DNA nanosphere sequencing and so on according to different sequencing principles. NGS enables the sequencing of up to millions of target nucleic acids at once, provides abundant variation information in short time in a cost-efficient way. Highlighting the characteristics of high output and high resolution, NGS has drawn more and more attention in multiple signaling pathways and target studies of cancer.

The preparation of sample library relies on specific modified primers and RingCap® mediated amplification technology with the employment of PCR apparatus. Specific modified primers enable the precise PCR amplification of target sequences, RingCap® mediated amplification allows terminal modification of the products with specific sequences. With the combination of particular PCR program and Ring-Cap polymerase, library preparation of target sequences could be achieved on common PCR apparatus before they are ready for high-throughput sequencing.

Kit Contents

Table 1. Kit Contents

NO.	Content Name	Components	Strip Color	16 Tests/Kit			32 Tests/Kit			Note
				Volume	Quantity	8-Tube Strip	Volume	Quantity	8-Tube Strip	
1	**EC-1 PCR Strip**	Primer, dNTPs, Mg^{2+}, PCR buffer	Blue	20 μL	16 Tubes	2 Strips	20 μL	32 Tubes	4 Strips	Each tube contains the same reaction
2	**EC-2 PCR Strip**	Primer, dNTPs, Mg^{2+}, PCR buffer	Pink	20 μL	16 Tubes	2 Strips	20 μL	32 Tubes	4 Strips	Each tube contains the same reaction
3	**Index 1-8 Reaction Strip**	Index primer, Mg^{2+}, dNTPs, PCR buffer	Purple	20 μL	8 Tubes	1 Strip	20 μL	8 Tubes	1 Strip	Each tube represents an Index number
4	**Index 9-16 Reaction Strip**	Index primer, Mg^{2+}, dNTPs, PCR buffer	Green	20 μL	8 Tubes	1 Strip	20 μL	8 Tubes	1 Strip	Each tube represents an Index number
5	**Index 17-24 Reaction Strip**	Index primer, Mg^{2+}, dNTPs, PCR buffer	White	——	——	——	20 μL	8 Tubes	1 Strip	Each tube represents an Index number
6	**Index 25-32 Reaction Strip**	Index primer, Mg^{2+}, dNTPs, PCR buffer	Yellow	——	——	——	20 μL	8 Tubes	1 Strip	Each tube represents an Index number
7	**RingCap-Taq（1#）**	Taq enzyme	——	15 μL	1 Tube	——	15 μL	2 Tubes	——	——
8	**EC Negative Control**	Wild type DNA	——	50 μL	1 Tube	——	50 μL	1 Tube	——	——
9	**EC Positive Control**	DNA with specific mutation sequence, wild-type DNA	——	50 μL	1 Tube	——	50 μL	1 Tube	——	——

Note 1: In Index reaction strips, different Index numbers respectively contain 32 different IlIDx recognition sequences (see Appendix Table 3); the reagents have been pre-packaged in 8-Tube strips; the left oblique position of the cap of the strip is oriented in the forward direction, from left to right followed by Index 1, 2, 3, 4, 5, 6, 7, 8 (Figure 1).

Note 2: The contents of different batches of reagents cannot be mixed.

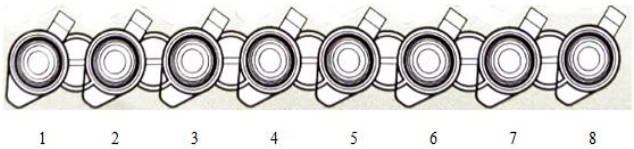

Figure 1 Index numbers of 8-tube strips

Additional required Equipment and Materials

1. Microvolume ultraviolet-visible spectrophotometer
2. Fluorometer: Quantus™ Fluorometer (Promega, Cat. No. E6150) or Qubit™4.0 Fluorometer (Thermo Fisher Scientific, Cat. No. Q33238)
3. Magnetic rack
4. Nucleic acids extraction kit: Nucleic Acid Extraction Kit (FFPE DNA) (Xiamen Spacegen Co., Ltd, Cat. No. SPG-HSD001) or Nucleic Acid Extraction Kit (Micro DNA) (Xiamen Spacegen Co., Ltd, Cat. No. SPG-HSMD001/002).
5. Quantification kit of nucleic acids: QuantiFluor® dsDNA System (Promega, Cat. No. E2670) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851/Q32854)
6. Magnetic beads: SG Pure Beads (Xiamen Spacegen Co., Ltd, Cat. No. SPG-PB001/002) or AMPure XP (Beckman Coulter, Cat. No. A63880/A63881/A63882) or HighPrep™ PCR (MagBio, Cat. No. AC-60005/ AC-60050/ AC-60250/ AC-60500)
7. Sequencing reagents: selecting the corresponding sequencing reagent according to the gene sequencer
8. Illumina PhiX Control V3 (Illumina, Cat. No. FC-110-3002)
9. Nuclease-free pipette tips with filter

10. TE buffer (pH 8.0)
11. Nuclease-free water
12. Absolute ethanol (Analytical Grade)

Applicable Instruments

1. Library preparation PCR apparatus: ABI 9700, ABI 2720, ABI MiniAmp, ABI Veriti, etc.
2. Sequencing instruments: Illumina sequencing instruments (Miseq, NextSeq 500/550, Miniseq,etc).

Transportation, Stability and Storage

1. Storage Condition: Store the kit away from light at -15℃ to -25℃, valid for 12 months. Once opened, reagents can be stored in their original packaging at -15℃ to -25℃ 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℃.
3. Check labels for production date and expiration date of the kit.

Specimen Material

The quality of the DNA to be detected is critical. Please collect samples according to the following recommended sample types, and then perform DNA extraction:

1. Recommended sample types: FFPE tissue or fresh tissue.
2. FFPE and fresh tissue samples: The diameter of fresh tissue shouldn't be less than 5 mm, and ensure that at least 20% of the collected pathological tissue were tumor lesions; it is recommended to choose FFPE samples that have not been stored for more than 2 years and at least 20% of the collected pathological tissue were tumor lesions, and use no less than 8 pieces of 5 μm section or 5 pieces of 10 μm section for nucleic acid extraction.
3. Commercial kits is recommended to extract genomic DNA from the samples. Assess the quality of sample DNA with an microvolume ultraviolet-visible spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.7-2.2. Use a fluorometer to determine the DNA concentration, the DNA concentration should be > 5 ng/μL, and the total amount of DNA should be > 50 ng. Once the DNA quality or quantity was not conformed with the above requirements, re-extract DNA with new and/or larger input. Proceed to library construction or store the DNA/cDNA at -15℃ to -25℃ for no more than 12 months.

Experimental Procedure

Note: Parallel library construction of **EC Positive Control** (PC) and **EC Negative Control** (NTC) with the tested sample is suggested.

I. Library Enrichment

1. Reagent preparation: Thaw the **EC-1 PCR Strip** (Blue) and **EC-2 PCR Strip** (Pink) at room temperature until no ice is present in the tubes, briefly centrifuge the strip before use. Place the **RingCap-Taq (1#)** on ice after centrifugation.
2. Sample preparation: Dilute sample DNA to 5 ng/μL with TE buffer (pH 8.0) based on the effective DNA concentration measured by the fluorometer, and the volume ≥ 10 μL.
3. Enriching reaction for EC PCR Strips
   (1) Add 0.5 μL of **RingCap-Taq (1#)** to 10 μL of the DNA Sample, EC PC and EC NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **EC-1 PCR Strip** (Blue) and **EC-2 PCR Strip** (Pink), and sequentially add 5 μL of the template prepared above (1) into the respective tube, cap the tubes carefully.
   (3) Centrifuge the tubes slightly and avoid creating air bubbles.
4. Load the PCR strip tubes above into the thermal cycler; then set up and run the program according to Table 2.

Table 2 PCR amplification procedure

Step	Temperature	Time	Cycle Number
Pre-denaturation	98℃	2 minutes	1
Denaturation	98℃	15 seconds	15

Annealing	65℃	4 minutes	
Hold	10℃	2 minutes	1

Note: Proceed to "Purification of Enriching Products", or store the products at 2-8℃ within 8 hours or at -15℃ to -25℃ within 24 hours. Storing for more than 24 hours is not suggested.

II. Purification of Enriching Products

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

1. Transfer all of the PCR enrichment product of **EC-1 PCR strip** and **EC-2 PCR strip** each to new 1.5 mL centrifuge tube, add 50 µL (1×sample volume) of magnetic beads to each tube, pipet up and down 5 times to mix magnetic beads suspension thoroughly with the product.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the tubes on a magnetic rack for 2 minutes until the solution becomes clear, carefully remove and discard the supernatant without disturbing magnetic beads.
   Note: The magnetic beads contain amplified library and should not be discarded.
4. Add 150 µL freshly prepared 70% ethanol to each tube, rotate the tubes clockwise or counterclockwise five times. Place the tubes on the magnetic rack for 2 minutes until the solution becomes clear, carefully remove and discard the supernatant without disturbing magnetic beads.
5. Repeat step 4 one more time for a second wash.
6. Remove all the ethanol from the tubes, and keep the tubes on the magnetic rack for 5 minutes at room temperature to air-dry the magnetic beads (avoid over-dry).
7. Remove the tube from the magnetic rack, add 35 µL of TE buffer (pH 8.0) to each tube to fully infiltrate the magnetic beads, and vortex thoroughly (or mix by pipetting at least half the total volume up and down at least 5 times), briefly centrifuge to collect the droplets.
8. Incubate the mixture for 5 minutes at room temperature.
9. Place the tubes on the magnetic rack for 2 minutes until the solution becomes clear, carefully transfer and store the supernatant (i.e. purified product), store at -15℃ to -25℃ or proceed to “Library Preparation”.

III. Library Preparation

Note: Use different Index for different samples.

1. Reagent preparation: Thaw the **Index Reaction Strip** based on DNA amount at room temperature until no ice is present in the tubes, briefly centrifuge the strip before use. Place the **RingCap-Taq (1#)** on ice after centrifugation.
2. Construction reaction
   (1) Add 0.25 µL of **RingCap-Taq (1#**) to 5 µL of the purified products of DNA Sample, EC PC and EC NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **Index Reaction Strip**, and sequentially add 5 µL of the template prepared above (1) into the respective tube, cap the tubes carefully.
   (3) Centrifuge the tubes slightly and avoid creating air bubbles.
3. Load the Index reaction strip tubes above into the thermal cycler; then set up and run the program according to Table 3.

Table3 PCR Amplification Procedure

Step	Temperature	Time	Cycle Number
Pre-denaturation	98℃	2 minutes	1
Denaturation	98℃	15 seconds	25
Annealing	65℃	4 minutes	
Hold	10℃	2 minutes	1

Note: Proceed to "Library Purification", or store the products at 2-8℃ within 8 hours or at -15℃ to -25℃ within 24 hours. Storing for more than 24 hours is not suggested.

IV. Library Purification

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

1. Transfer 25 μL of PCR product each to new 1.5 mL centrifuge tube, add 25 μL (1×sample volume) of magnetic beads to each tube, pipet up and down 5 times to mix the bead suspension thoroughly with the product.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the tubes on a magnetic rack for 2 minutes until the solution becomes clear, carefully remove and discard the supernatant without disturbing magnetic beads.
   Note: The magnetic beads contain amplified library and should not be discarded.
4. Add 150 μL of freshly prepared 70% ethanol to each tube, rotate the tubes clockwise or counterclockwise five times. Place the tubes on the magnetic rack for 2 minutes until the solution becomes clear, carefully remove and discard the supernatant without disturbing magnetic beads.
5. Repeat step 4 one more time for a second wash.
6. Remove all the ethanol from the tubes, and keep the tubes on the magnetic rack for 5 minutes at room temperature to air-dry the magnetic beads (avoid over-dry).
7. Remove the tubes from the magnetic rack, add 35 μL of TE buffer (pH 8.0) to each tube to fully infiltrate the magnetic beads, and vortex thoroughly (or mix by pipetting at least half the total volume up and down at least 5 times), briefly centrifuge to collect the droplets.
8. Incubate the mixture for 5 minutes at room temperature.
9. Place the tubes on the magnetic rack for 2 minutes until the solution becomes clear, carefully remove and store the supernatant (i.e. library) or store at -15℃ to -25℃ or proceed to “Library Quantification and Dilution”.

V. Library Quantification and Dilution

1. Quality control of sample library: Bioanalyzer is recommended for the quality control of library fragments; for NTC library, PC library and sample libraries, the main fragments should be in 250-350 bp. A fluorometer is recommended to measure the concentration of the library.
2. Fluorometer quantification kit is recommended to measure the concentration of the sample library and should be ≥ 1 ng/μL.
3. 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.

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

4. Per the concentration measured, dilute the sample library to 4 nM with nuclease-free water.
5. Store undiluted libraries at -15℃ to -25℃ for up to 7 days; the mixture of diluted libraries is suggested to be used right after it is ready.

VI. Sequencing

1. The data volume of each sample is recommended to be not less than 0.4 Gb. The Phix Control V3 is recommended to account for 5-15%.
2. Prepare fresh 0.2 M NaOH: add 2 μL 10M NaOH to 98 μL purified water and mix thoroughly.
3. Based on the data volume of flowcell to decided the number of detected sample, and mix each sample in equal proportions, gain a 4 nM mixed library.
4. Denaturation and dilution of the library: draw 5 μL 4nM mixed library to a new 1.5 mL centrifuge tube, and add 5 μL 0.2 M NaOH, then mix the mixture thoroughly and gently, incubate the mixture for 5 minutes at room temperature.Draw 5 μL denatured library to a new 1.5 mL centrifuge tube and add 495 μL HT1, gain the 20 pM library.
5. Denaturation and dilution of the PhiX: draw 1 μL 10 nM PhiX Control V3 to a new 1.5 mL centrifuge tube, add 1.5 μL HT1 to dilute the PhiX Control V3 to 4 nM, then add 2.5 μL 0.2 M NaOH, then mix the mixture thoroughly and gently, incubate the mixture for 5 minutes at room temperature. Then add 495 μL HT1, gain the 20 pM PhiX Control V3.
6. Take the NextSeq 500/550 as an example, follow the table 4 to mix and dilute the final library (the Phix Control V3 account for 10%).

Table 4 Mix and dilution of the sequencing final library with NextSeq 500/550

Instrument model	Sequencing concentration	Volume of mixed library（20 pM）	Volume of PhiX（20 pM）	Volume of HT1	Volume of final library
NextSeq 500/550	1.3 pM	76.05 μL	8.45 μL	1215.5 μL	1300 μL

7. Thaw reagent cartridge at room temperature, and flip 10 times to mix the Reagent Cartridge.

8. Use a new 1 mL pipette tip to pierce the sealing foil of the library loading port on the reagent cartridge, load all of the final library from step 5 into the library loading port, be careful not to contact with sealing foil.
9. Follow the interface prompts on the sequencer used to perform sequencing flowcell and reagent cartridge loading operation, perform the sequencing after the sequencer self-test is completed.
10. Clean and shut down the instrument after the sequencer sequencing is completed.

Note: For other gene sequencers, perform sequencing according to the corresponding standard operating procedures.

VII. Bioinformatics Analysis

1. Use Illumina Sequencing Analysis Viewer v1.9.1 software to perform quality analysis of the sequencing data. The Q30 data quality should be higher than 75%.
2. Upload the FastQ file to the server. Perform data quality control, sequence alignment, mutation analysis, mutation annotation, copy number variation analysis and instability analysis of micro satellite sites by the Clinical NGS Data Analysis Software (hereinafter referred to as the analysis software) of Xiamen Spacegen Co., Ltd.
3. Use the quality control module of the analysis software (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 software (based on BWA v0.7.17 and SAMtools v1.9) to compare the filtered FastQ files to the human reference genome (hg19 version), generating BAM files and BAI files.
5. Mutation analysis: Use the mutation detection module of the analysis software (based on Pisces v5.2.9) to detect point mutations and insertion deletion mutations in the target region "SG_EC_target.region_V1.0.bed".
6. Mutation annotation: Use the variant annotation module of the analysis software (based on ANNOVAR v20180426) toannotate the identified point mutations and insertion deletion mutations in accordance with the AMP-ASCO-CAP 2017 guidelines.
7. Copy number variation analysis: Use the copy number variation analysis module of the analysis software (based on SG-CNV v1.1), by comparing the difference in sequencing depth between the tested sample and the normal sample (baseline), obtain the Ratio of the tested gene through statistical analysis, and the copy number variation of the gene is determined based on the Ratio.
8. Analysis of micro satellite instability: Use the MSI analysis module (based on SG-MSI v1.2) to analyze each micro satellite site to asses microsatellite instability status with chi-square test comparing read length distribution in tumor and normal samples(baseline) and count the MSI-Score.

Data Analysis

1. The data quality control standards: The Ontarget Ration of the sequencing data is recommended to be ≥ 80% while the Mean Depth is recommended to be ≥ 2000 ×, the Uniformity is should be ≥ 75%.
2. Positive judgment: In the somatic mutation analysis results, if the mutation frequency is not less than 5%, it is judged as positive. otherwise, it is judged as negative or the detection is lower than the detection limit of the kit based the effective depth of sequencing is not less than 100 ×.
3. The result of CNV: The Total Reads of the target genes accounted for the target region divided by the average of the percentage of the target gene with baseline samples, followed the CNV-Ratio result is obtained. If the CNV-Ratio≥2, it is judged as copy number amplifications. If the CNV-Ratio <2, it is judged as negative or below the detection limit.
4. Analysis of the unstable state of micro satellites, require the qualified quality control of the microsatellite sites numbers should be≥ 20, according to MSI-Score to judge the unstable state of micro satellites:
   Micro satellite stable (MSS): MSI-Score < 0.3.
   Micro satellite instability (High-frequency MSI, MSI-H): MSI-Score ≥ 0.3.

Interpretation of Results

1. For the sample libraries, the target fragment should be in 250-350 bp, each amplicon should have coverage, as well as On Target should be ≥ 80%, Uniformity should be ≥ 75%, moreover, Mean Depth ≥ 2000×; otherwise the results of the sample DNA is invalid.
2. For the NTC library, the target fragment should be in 250-350 bp, as well as On Target should be ≥ 80%, Uniformity should be ≥ 75%, moreover, Mean Depth ≥ 2000×; The somatic mutation results is judged as negative, and the microsatellite unstable site is not detected, the library quality control of negative control is considered to be as qualified. If the mutation result is positive or the microsatellite status is instability, this indicates that there may be a source of DNA contamination in the environment.

3. For the PC library, the target fragment should be in 250-350 bp, as well as On Target should be ≥ 80%, Uniformity should be ≥ 75%, moreover, Mean Depth ≥ 2000×; The qualified detection results should be consistent with the information of the positive control table (Appendix Table 2.) Otherwise, the detection is invalid.

Limitation of the Kit

1. The detection results are for research use only. For mutation sites that were not included in the kit, or the DNA extracted from FFPE samples were stored longer than required, the results shall not be interpreted by the instruction.
2. The negative results cannot exclude the mutations. Too few tumor cells, excessive degradation, or the DNA concentration is below the detection limit can also cause a negative result.
3. Unreasonable sample collection, transportation, processing, improper operation and the experimental environment may lead to false negative or positive results.
4. Tumor tissues (cells) may have large heterogeneity, different test results may be obtained by sampling different parts.
5. The detection of gene copy number variation is easily affected by multiple factors such as tumor cell proportion, sequencing uniformity and chromosome ploidy.

Performance characteristics

1. The kit should be of neat appearance, clear labels, and of no leakage. When unfrozen, the reagents shall be clear, without sediments.
2. There are 24 copies of enterprise positive reference were tested, 16 national positive reference products were tested for micro satellite instability (MSI), and the compliance rate of positive reference products was 100%.
3. There are 8 copies of negative reference and stable micro satellites within the detection range and 3 national negative reference products with micro satellite instability (MSI) were tested. The coincidence rate of the negative reference products was 100%.
4. Detects gene mutations as low as 5% in a 25 ng DNA sample.
5. The detection limit of micro satellite instability is 5% of tumor cell DNA content.
6. There are 2 copies of the enterprise's positive repeatable reference were tested for 10 times, and the test results were all the corresponding mutation types or micro satellite instability; 1 copy of the company's negative repeatable reference were tested, repeated 10 times, The results were all mutation negative and micro satellite stable.
7. If the concentration of hemoglobin, paraffin, formalin, and ethanol remaining in the sample nucleic acid is not higher than 2 g/L, 1%V/V, 0.005%V/V, 1%V/V, the results will not be affected.

Warnings and Precautions

1. Please read this instruction carefully in prior to experiments.
2. Conduct experiments abide by laboratory regulations to reduce cross-contamination of products or reagents; divide experiment areas into different function zones if possible.
3. Clean experiment areas before experiment with 10% hypochlorous acid followed by water rinsing. Sterilize the environment and pipettes with 10% hypochlorous acid, 75% ethanol, or UV radiation.
4. Avoid using peripheral wells of PCR instrument; vacate holes or columns between samples to avoid cross-contamination.
5. Testing results might be influenced by sample sources, sampling process, sample quality, carriage conditions, sample handling, etc; also might it be limited by the quality of DNA, instrument types, operating environment, and the limitation of current molecular biotechnology, all of which may lead to false positive/ negative results. The users should thoroughly be informed of potential errors as well as the limitation of accuracy.
6. Avoid unnecessary freezing-thawing the reagents, the reagents were allowed to undergo no more than 5 freeze-thaw cycles.
7. The quality of DNA matters experimental results to a great extent, hence, proceed to the next step as soon as possible or store at the recommended temperature when finish the extraction.
8. Do not substitute any original reagents contained in the kit. Do not mix reagents with different lots.
9. The use of filter tips is highly recommended to avoid false-positive results caused by contamination of reagents.
10. Be cautious of contamination from external DNA; use specific pipettes and tips for preparation of reagents and template addition. The reagent preparation area should be separated from the template adding area.
11. All reagents in use have potential hazard. For first-use of this kit, you may receive training by our technical supports. All used contents of the kit should be considered as clinical dessert and should be disposed properly.

12. All samples including positive control in the kit should be considered potential infectious substances. They should be handled carefully.

Symbols

Symbol	Symbol definition
	Indicates the need for the user to consult the instructions for use.
	Indicates a medical device that is intended to be used as an in vitro diagnostic medical device.
	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 devices hould be kept dry.
	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

Lotus NL B.V.
Address:Koningin Julianaplein 10, 1e Verd, 2595AA, The Hague, Netherlands.
E-mail: peter@lotusnl.com

Manufacturer: XIAMEN SPACEGEN CO., LTD.
Address: 4th floor, No.2041 Xizhou Road, Xike Town, 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

Appendix table 1

The detection range of this kit

Gene/Item	Detection Range
TP53	Complete coding region (CDS) and exon-intron junction
POLE	Exons 9-14 and the exon-intron junction
MLH1	Complete coding region (CDS) and exon-intron junction
MSH2	Complete coding region (CDS) and exon-intron junction
MSH6	Complete coding region (CDS) and exon-intron junction
PMS2	Complete coding region (CDS) and exon-intron junction
EPCAM	Complete coding region (CDS) and exon-intron junction
PTEN	Complete coding region (CDS) and exon-intron junction
CTNNB1	Exons 3
KRAS	Exons 2-4
PIK3CA	Exons 10, 14, 21
HER2	Exons 19, 20, 21
MSI	34 micro satellite loci

Appendix table 2

Positive control information of this kit

Gene Name	Base Mutation	Amino Acid Mutation	Cosmic ID	Mutation Type
POLE	c.1366G>C	p.A456P	COSM937318	Point Mutation
MSH6	c.3261del	p.F1088Sfs*2	COSM330655	Deletion Mutation
MLH1	c.1513del	p.S505Vfs*3	COSM26802	Deletion Mutation
PTEN	c.464A>G	p.Y155C	COSM5144	Deletion Mutation
PTEN	c.955_958del	p.T319*	COSM4898	Deletion Mutation
TP53	c.377A>G	p.Y126C	COSM11517	Point Mutation
TP53	c.267_268insC	p.S90Lfs*59	COSM131024	Insertion Mutation
Microsatellite Instability：MSI-H				

Appendix table 3.

32 different IllDx recognition sequences

Color	Number	i7 Sequence	i5 Sequence
Purple	Index_1	TAAGGCGA	CTCTCTAT
	Index_2	CGTACTAG	TATCCTCT
	Index_3	AGGCAGAA	GTAAGGAG
	Index_4	TCCTGAGC	ACTGCATA
	Index_5	GGACTCCT	AAGGAGTA
	Index_6	TAGGCATG	CTAAGCCT
	Index_7	CTCTCTAC	CGTCTAAT
	Index_8	CGAGGCTG	TCTCTCCG
Green	Index_9	TAAGGCGA	TATCCTCT
	Index_10	CGTACTAG	GTAAGGAG
	Index_11	AGGCAGAA	ACTGCATA
	Index_12	TCCTGAGC	AAGGAGTA
	Index_13	GGACTCCT	CTAAGCCT
	Index_14	TAGGCATG	CGTCTAAT

Color	Number	i7 Sequence	i5 Sequence
White	Index_17	TAAGGCGA	GTAAGGAG
	Index_18	CGTACTAG	ACTGCATA
	Index_19	AGGCAGAA	AAGGAGTA
	Index_20	TCCTGAGC	CTAAGCCT
	Index_21	GGACTCCT	CGTCTAAT
	Index_22	TAGGCATG	TCTCTCCG
	Index_23	CTCTCTAC	CTCTCTAT
	Index_24	CGAGGCTG	TATCCTCT
Yellow	Index_25	TAAGGCGA	ACTGCATA
	Index_26	CGTACTAG	AAGGAGTA
	Index_27	AGGCAGAA	CTAAGCCT
	Index_28	TCCTGAGC	CGTCTAAT
	Index_29	GGACTCCT	TCTCTCCG
	Index_30	TAGGCATG	CTCTCTAT

	Index_15	CTCTCTAC	TCTCTCCG
	Index_16	CGAGGCTG	CTCTCTAT

	Index_31	CTCTCTAC	TATCCTCT
	Index_32	CGAGGCTG	GTAAGGAG