CE

RingCap®

Oncology Multi-Gene Mutations Detection Kit

High-Throughput Sequencing

Instruction for Use (Illumina)

Product Name

Oncology Multi-Gene Mutations Detection Kit (High-Throughput Sequencing)

Packing Specification

16 Tests/kit, 32 Tests/kit

Intended Use

The kit is intended for the detection of gene somatic mutations (see Appendix Table 1, 2) in peripheral blood or FFPE pathological tissue collected from patients with non-small cell lung cancer or colorectal cancer. The results are indicated only to aid in the individualized therapy of non-small cell lung cancer or colorectal cancer patients. The results shall not be regarded as the only evidence to guide whether a patient suits individualized therapy; determinants such as, but not limited to patients' condition, drug indications, therapeutic response and other laboratory detection indexes should also be considered before making comprehensive judgments.

The kit facilitates the detection of 484 somatic mutations of 13 genes (See Appendix Table 2), including single base mutations, insertions, deletions, and gene fusions [1-8]. The correlations between gene mutations and specific target drugs were mainly from literatures and were generally recognized by clinical practice [5-10].

Technological Principle

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 one time, provides abundant variation information in short time and at relatively low cost. 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 feasibility of NGS-based multi-pathways/targets detection as an aid in the diagnosis of disease has been supported by numerous clinical trials (e.g. Lung-MAP1, CRUK, WIN Consortium, and NCI-MATCH) [1-6].

The construction 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 construction of target sequences could be achieved on common PCR apparatus before they are ready for high-throughput sequencing.

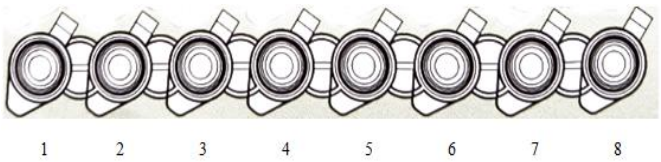
Kit Contents

Table 1 Kit contents

No.	Content Name	Main Content	Strip Color	16 Tests/Kit			32 Tests/Kit			Note
				Volume	Quantity	8-Tube Strip	Volume	Quantity	8-Tube Strip	
1	**Onco-DNA PCR Strip**	Primer, dNTPs, Mg^{2+}, Buffer	Blue	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.
2	**Onco-RNA PCR Strip**	Primer, dNTPs, Mg^{2+}, Buffer	Pink	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.
3	**UDI 1-8 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Purple	20 μL	8 tubes	1 strip	20 μL	16 tubes	2 strips	Each tube represents an UDI.
4	**UDI 9-16 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Green	20 μL	8 tubes	1 strip	20 μL	16 tubes	2 strips	Each tube represents an UDI.
5	**UDI 17-24 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	White	20 μL	8 tubes	1 strip	20 μL	16 tubes	2 strips	Each tube represents an UDI.
6	**UDI 25-32 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Yellow	20 μL	8 tubes	1 strip	20 μL	16 tubes	2 strips	Each tube represents an UDI.
7	**RingCap-Taq (1#)**	Taq enzyme	——	20 μL	1 tube	——	40 μL	1 tube	——	——

8	**Onco-DNA Negative Control**	Wild type DNA	——	20 μL	1 tube	——	20 μL	1 tube	——	——
9	**Onco-RNA Negative Control**	Wild type cDNA	——	20 μL	1 tube	——	20 μL	1 tube	——	——
10	**Onco-DNA Positive Control**	Mutation type DNA	——	20 μL	1 tube	——	20 μL	1 tube	——	——
11	**Onco-RNA Positive Control**	Mutation type cDNA	——	20 μL	1 tube	——	20 μL	1 tube	——	——

Note 1: In UDI reaction strips, different UDI numbers respectively contain different UDI recognition sequences (see Appendix Table 5,6). 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 UDI 1, 2, 3, 4, 5, 6, 7, 8 (Figure 1).

Figure 1. UDI numbers of 8-Tube Strips

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

Additional required Equipment and Materials

1. Nucleic acids extraction kit: Nucleic Acid Extraction Kit (FFPE DNA+RNA) (Xiamen Spacegen Co., Ltd, Cat. No. SPG-HSDR001R/002R) or Nucleic Acid Extraction Kit (Plasma DNA) (Xiamen Spacegen Co., Ltd, Cat. No. SPG-HSPD001R) or Nucleic Acid Extraction Kit (Peripheral blood RNA Centrifugal column method) (Xiamen Spacegen Co., Ltd, Cat. No. SPG-HSBR001R)
2. RNA reverses transcription kit: Super Script™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. 11754-050)
3. Quantification kit of nucleic acids: Quanti Fluor® dsDNA System (Promega, Cat. No. E2670) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851/Q32854), Qubit® ssDNA Assay Kit (Alternatively) (Thermo Fisher Scientific, Cat. No. Q10212)
4. Fluorometer: Quantus™ Fluorometer (Promega, Cat. No. E6150) or Qubit™4.0 Fluorometer (Thermo Fisher Scientific, Cat. No. Q33238)
5. Magnetic beads: SG Pure Beads (Xiamen Spacegen Co., Ltd, Cat. No. SPG-PB001R/002R) or HighPrep™ PCR (MagBio, Cat. No. AC-60005/ AC-60050/ AC-60250/ AC-60500)
6. Sequencing reagents and corollary reagents to be purchased separately: Selecting the corresponding sequencing reagent according to the gene sequencer
   (1) Illumina corollary reagents: PhiX Control V3 (Illumina, Cat. No. FC-110-3001)
   (2) MGI corollary reagents: MGIEasy universal library conversion kit (APP-A) (MGI, Cat. No. 1000004155), High throughput sequencing primer kit (App-C) (Alternatively) (MGI, Cat. No. 1000027472)
7. Magnetic rack
8. Microvolume UV-visible spectrophotometer
9. Ethanol absolute (Analytical Grade)
10. TE Buffer (pH 8.0)
11. Nuclease-Free Water
12. Nuclease-Free pipette tips with filter

Transportation, Stability and Storage

1. Storage Condition: Store the kit away from light at -15℃ to -25℃, valid for 9 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 the production date and expiration date of the kit.

Applicable Instruments

1. Library preparation PCR apparatus: ABI 9700, ABI 2720, ABI Veriti, ABI Mini Amp, etc.
2. Sequencing instruments:
   (1) Illumina sequencing instruments (Miseq, NextSeq 500/550, Miniseq, etc)
   (2) MGI sequencing instruments (MGISEQ-2000, DNBSEQ-G99RS, etc)

Specimen Material

The quality of the nucleic acids to be detected is critical. Please collect samples according to the following recommended sample types:

1. Recommended sample types: FFPE, peripheral blood.
2. FFPE samples: It is recommended to choose FFPE samples that have not been stored for more than 2 years and at least 30% of the collected pathological tissue is tumor lesions, and use no less than 8 pieces of 5 μm section or 5 pieces of 10 μm section for nucleic acids extraction.
3. Peripheral blood samples: Peripheral blood should be collected with a cell-free DNA blood-collecting vessel with volumes no less than 10 mL.

Experimental Procedure

Note: Parallel library construction of **Onco-DNA Positive Control** (Onco-DNA PC), **Onco-RNA Positive Control** (Onco-RNA PC) and **Onco-DNA Negative Control** (Onco-DNA NTC) and **Onco-RNA Negative Control** (Onco-RNA NTC) with the tested sample is suggested.

I. Library Enrichment

1. Reagent preparation: Thaw the **Onco-DNA PCR Strip** (Blue) and **Onco-RNA PCR Strip** (Pink) as needed 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:
   (1) Nucleic acid extraction and quality control:
      (a) Commercial nucleic acids extraction kit is recommended to extract genomic DNA from the samples. Assess the quality of sample DNA with a Microvolume UV-visible spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.8-2.2, quantify sample DNA with a Fluorometer, the concentration should be ≥2 ng/μL, the total amount of DNA should be ≥10 ng. Once the DNA quality or quantity is not conformed with the above requirements, re-extract DNA with new and/or larger input. DNA is recommended to library construction immediately or store at -15℃ to -25℃ for no more than 12 months.
      (b) Commercial nucleic acids extraction kit is recommended to extract genomic RNA from the samples. Assess the quality and quantity of sample RNA with a Microvolume UV-visible spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.8-2.3, the concentration should be ≥20 ng/μL, the total amount of RNA should be ≥100 ng. Once the RNA quality or quantity is not conformed with the above requirements, re-extract RNA with new and/or larger input. Reverse transcription is performed immediately after RNA extraction. cDNA is recommended to library construction immediately or store at -15℃ to -25℃ for no more than 12 months.
   (2) DNA Sample: Dilute DNA sample to 2 ng/μL with TE Buffer (pH 8.0) based on the effective DNA concentration measured by the Fluorometer, and the volume ≥5 μL.
   (3) cDNA Sample: cDNA sample that after reverse transcription, and the volume ≥5 μL.
3. Enriching reaction for DNA
   (1) Add 0.25 μL of **RingCap-Taq (1#)** to 5 μL of the DNA Sample, Onco-DNA PC and Onco-DNA NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **Onco-DNA PCR 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.
4. Enriching reaction for cDNA
   (1) Add 0.25 μL of **RingCap-Taq (1#)** to 5 μL of the cDNA Sample, Onco-RNA PC and Onco-RNA NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **Onco-RNA PCR 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.

5. Load the PCR centrifuge 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	4℃	∞	1

Note: Proceed to "Purification of Enriching Products", or store the products at 2℃ to 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 25 µL of PCR enrichment product of **Onco-DNA PCR Strip** each to a new 1.5 mL centrifuge tube, add 37.5 µL (1.5×sample volume) of magnetic beads to each tube, pipet up and down 5 times to mix magnetic beads suspension thoroughly with the product.
2. Transfer 25 µL of PCR enrichment product of **Onco-RNA PCR Strip** each to a new 1.5 mL centrifuge tube, add 37.5 µL (1.5×sample volume) of magnetic beads to each tube, pipet up and down 5 times to mix magnetic beads suspension thoroughly with the product.
3. Incubate the mixture for 5 minutes at room temperature.
4. 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.

5. 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.
6. Repeat step 5 one more time for a second wash.
7. 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).
8. 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.
9. Incubate the mixture for 5 minutes at room temperature.
10. 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 UDI for different DNA or cDNA samples.

1. Reagent preparation: Thaw the **UDI Reaction Strip** based on samples 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 for DNA
   (1) Add 0.25 µL of **RingCap-Taq (1#)** to 5 µL of the purified products of DNA Sample, Onco-DNA PC and Onco-DNA NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **UDI 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. Construction reaction for cDNA
   (1) Add 0.25 µL of **RingCap-Taq (1#)** to 5 µL of the purified products of cDNA Sample, Onco-RNA PC and Onco-RNA NTC, vortex slightly to mix, and then centrifuge briefly.
   (2) Gently remove the cap of the **UDI Reaction Strip**, 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 **UDI Reaction Strip** tubes above into the thermal cycler, then set up and run the program according to Table 3.

Table 3. PCR Amplification Procedure

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

Note: Proceed to "Library Purification" or store the products at 2℃ to 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 a new 1.5 mL centrifuge tube, add 37.5 μL (1.5×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.
4. 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 next step.

V. Library Quantification

Bioanalyzer is recommended for the quality control of library fragments. For DNA NTC libraries and DNA PC library and all sample DNA libraries, the target fragments should be in 200-300 bp. For RNA NTC libraries and RNA PC library and all sample cDNA libraries, the target fragments should be in 200-300 bp. Fluorometer quantification kit is recommended to measure the concentration of sample library and should be more than 0.5 ng/μL.

VI. Library dilution, mixing and sequencing

1. Illumina sequencing platform
   (1) According to the library concentration measured by the Fluorometer, use the following formula to convert the molar concentration of the library.

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

   (2) Per the concentration measured, dilute the sample library to 4 nM with Nuclease-Free Water.
   (3) The proportion of DNA and cDNA is 4:1 (Mix 20 μL of each DNA sample library with 5 μL of cDNA sample library).
   (4) Proceed sample dilution and denaturation according to the matching Illumina sequencing kit (refer to the operation manual of each equipment).
   (5) The concentration of Phix Control V3 is more than 5% (for example: If the loading volume is 600 μL, the volume occupied by Phix Control V3 should be more than 30 μL).
   (6) Library on-machine sequencing (according to the specification of instrument and matching reagent).

2. MGI sequencing platform

(1) The recommended amount of cyclic library input is 0.5 pmol, the loading ratio of DNA library and cDNA library is 4:1, and the required proportion of each library in the total library of 0.5 pmol is calculated, and then the required amount of each library is calculated according to the following formula:

$$\text{Library input (ng)} = \frac{\text{Library length (bp)} \times 650 \times \text{proportion\%}}{1000}$$

(2) According to the measured library concentration, the required input volume is calculated, and then mixed to obtain 0.5 pmol total library, the total volume is not more than 48 μL.

(3) Denaturation and cyclization of libraries according to the MGIEasy Universal Library Conversion Kit (App-A) (no need for split-conversion PCR, see the accompanying reagent manual for instructions).

(4) Proceed sample dilution and denaturation according to the matching sequencing kit (refer to the operation manual of each equipment).

(5) Library DNB preparation and on-machine sequencing (operation instructions refer to the accompanying reagent manual and instrument manual).

Note: 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.

VII. Bioinformatics Analysis

Transfer the Fastq files obtained by sequencing to the analysis server, perform data quality control, sequence alignment, mutation annotation, and gene fusion analysis-based on the Clinical NGS Data Analysis System of Xiamen Spacegen Co., Ltd.

Data Analysis

1. Results of DNA

(1) Standard of quality: For all sample DNA libraries, the target fragment should be in 200-300 bp, On Target should be ≥80%, Uniformity should be ≥75% and mean depth should be ≥5000×.

(2) Mutated positive judgement criterion: In the results of variation analysis, if effective depth is >500×. When there is COSMIC ID in the sample analysis, this mutation site is judged as positive mutation. Otherwise, it is judged as negative or below the detection limit.

2. Results of RNA

(1) Standard of quality: For all sample cDNA libraries, the target fragments should be in 200-300 bp. The value of "Total Reads" of at least 2 of the 5 internal control genes (HMBS, TBP, JUN, LRP1, and MRPL13) recorded in the file should be ≥200×, that guarantees the quality of RNA sample.

(2) Mutated positive judgement criterion: The interpretation mode of fusion mutation provided by this kit is "specific fusion site detection". The sequence data obtained from fusion transcription analysis is recorded in the format of several readings per target, which is different from other analysis platforms. The background signal level should be taken into account when interpreting the results:

(a) If the sample is not read in both positive and negative directions, indicate the specific fusion site is negative.

(b) If the sample is read in both positive and negative directions, and the Total Reads is less than 200×, indicates that the fusion close to the background signal has been detected, it is recommended to increase the input of the library; After re-detection, the Total Reads is still less than 200× indicate the specific fusion site is negative or lower than the detection limit.

(c) If the sample is read in both positive and negative directions, and Total Reads ≥200×, indicate the specific fusion site is positive.

Interpretation of Results

1. For the DNA negative control library, the target fragment should be in 200-300 bp, as well as On Target should be ≥80%, Uniformity should be ≥75%, moreover, mean depth ≥5000×. Otherwise, this test is invalidated.
2. For the RNA negative control library, the target fragments should be in 200-300 bp, it should more than 2 of the 5 internal control genes are all read, and the Total Reads ≥200×. Otherwise, this gene fusion test results are invalidated.
3. For the DNA positive control library, the target fragment should be in 200-300 bp, as well as On Target should be ≥80%, Uniformity should be ≥75%, moreover, mean depth ≥5000×. Otherwise, this test is invalidated.

4. For the RNA positive control library, the target fragments should be in 200-300 bp, it should more than 2 of the 5 internal control genes are all read, and the Total Reads ≥200×. Otherwise, this gene fusion test results are invalidated.
5. For the DNA sample libraries, the target fragment should be in 200-300 bp, each amplicon should have coverage, as well as On Target should be ≥80%, Uniformity should be ≥75%, moreover, mean depth ≥5000×. Otherwise, this mutation detection results are invalidated.
6. For the RNA sample libraries, the target fragments should be in 200-300 bp, it should more than 2 of the 5 internal control genes are all read, and the Total Reads ≥200×. Otherwise, this gene fusion test results are invalidated.
7. The grade of somatic variation based on the "Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists" jointly formulated by AMP/ASCO/CAP in 2017 could divide into 4 types:
   (1) Clear clinical significance: Diagnostic\prognostic marker of specific tumor or drugs recommended\approved in the professional guidelines.
   (2) Potential clinical significance: Diagnostic\prognostic marker of specific tumor or drugs that has level A evidence of another tumor in the multiple small research.
   (3) Unknown clinical significance: It is not found higher rates of variants in the general population and tumor databases, moreover, not has clear published evidence.
   (4) Harmless or may be harmless clinical significance: It is found higher rates of variants in the general population and not published evidence.

Limitation of the Kit

1. The detection results are for research use only. For mutation sites that are not included in the kit, or the nucleic acids extracted from samples are stored longer than required, the results shall not be interpreted by the instruction.
2. The negative results cannot exclude the mutations. For few tumor cells, excessive degradation, or the nucleic acids 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 tissue (cells) may have large heterogeneity, different test results may be obtained by sampling different parts.

Performance characteristics

1. The kit should be neat in appearance, clearly labels, and no leakage.
2. When unfrozen, the reagents shall be clear, without sediments.
3. Negative reference conformity rate should be 100%.
4. Positive reference conformity rate should be 100%.
5. The kit allows the detection of 5% of specific gene mutations in 10 ng DNA tissue samples.
6. The kit allows the detection of 20 copies/μL of fusion mutations in RNA tissue samples.

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 nucleic, 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 nucleic acids is crucial, and the quality control of DNA should be performed after extraction, proceed to further steps immediately or store properly at -15℃ to -25℃. RNA is recommended to be reverse transcript to cDNA before storage, and RNA without reverse transcription is recommended to be stored below-70℃.
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 nucleic. Ensure to add the nucleic 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% ethanol, or UV radiation after experiment.
10. All 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.
12. Avoid using peripheral wells of PCR instrument; vacate holes or columns between samples to avoid cross-contamination.

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 device should 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.

References

1. Mork TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009, 361 (10): 947-57.

2. Gazdar AF. Personalized medicine and inhibition of EGFR signaling in lung cancer. N Engl J Med. 2009, 361 (10): 1018-20.
3. Dancey JE. Epidermal growth factor receptor inhibitors in non-small cell lung cancer. Drugs. 2007, 67 (8): 1125-38.
4. Jemal A, Bray F, Center MM, el al. Global cancer statistics. CA Cancer J Clin. 2011, 61 (2): 69-90.
5. Soulières D, Greer W, Magliocco AM, et al. KRAS mutation testing in the treatment of metastatic colorectal cancer with anti-EGFR therapies. Curr Oncol. 2010, 17 Suppl 1: S31-40.
6. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenibin melanoma with BRAFV600E mutation. N Engl J Med. 2011, 364 (26): 2507-16.
7. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010, 11 (8): 753-62.
8. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007, 448 (7153): 561-6.
9. Ou SH, Tan J, Yen Y, et al. ROS1 as a 'druggable' receptor tyrosine kinase: lessons learned from inhibiting the ALK pathway. Expert Rev Anticancer Ther. 2012, 12 (4): 447-56.
10. Turke AB, Zejnullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 2010, 17 (1): 77-88.

EC REP

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Website: http://www.sspacegen.com

Appendix Table 1. Information of designated gene detected by the kit

No.	Name	Gene Type	Covered Exons	Number of Mutations
1	EGFR	DNA	18,19,20,21	102
2	KRAS		2,3,4	56
3	BRAF		15	41
4	PIK3CA		10,14,21	51
5	NRAS		2,3,4	34
6	HER2		19,20,21	16
7	MET		2,14,16,19,20	11
8	AKT1		2	1
9	c-KIT		9,11,13,17	100
10	PDGFRA		12,18	21
11	ALK	RNA	Fusion mutation	21
12	RET		Fusion mutation	15
13	ROS1		Fusion mutation	15

Appendix Table 2. COSM/COSF ID information of 484 mutations

No.	Gene	Covered Exons	COSMIC ID
1	NRAS	2	COSM577, COSM576, COSM573, COSM574, COSM575, COSM569, COSM570, COSM571, COSM567, COSM564, COSM565, COSM566, COSM12723, COSM561, COSM562, COSM563, COSM558
		3	COSM589, COSM585, COSM586, COSM587, COSM30646, COSM33693, COSM582, COSM583, COSM584, COSM12725, COSM579, COSM580, COSM581, COSM12730, COSM28673
		4	COSM4170228, COSM2717
2	PIK3CA	10	COSM759, COSM17442, COSM760, COSM762, COSM249872, COSM125370, COSM27133, COSM763, COSM12458, COSM27155, COSM764, COSM27374, COSM765, COSM6147, COSM766, COSM12459, COSM25041, COSM767, COSM24712
		14	COSM778, COSM5030972
		21	COSM12590, COSM771, COSM36285, COSM772, COSM21451, COSM36286, COSM17445, COSM29110, COSM25085, COSM12591, COSM12463, COSM29313, COSM773, COSM94984, COSM94985, COSM27134, COSM12592, COSM25086, COSM27273, COSM27156, COSM774, COSM775, COSM776, COSM94986, COSM94987, COSM24714, COSM36289, COSM12597, COSM777, COSM27158
3	PDGFRA	12	COSM21973, COSM741, COSM739, COSM28053, COSM12417, COSM12418
		18	COSM12405, COSM12396, COSM12398, COSM12397, COSM12406, COSM12401, COSM737, COSM12411, COSM736, COSM96892, COSM12408, COSM12400, COSM12407, COSM12402, COSM12399
4	c-KIT	9	COSM1326, COSM96885
		11	COSM23418, COSM1204, COSM1205, COSM1210, COSM1330, COSM1211, COSM1213, COSM1221, COSM1216, COSM1219, COSM1220, COSM24748, COSM1217, COSM1218, COSM1223, COSM1332, COSM1227, COSM1226, COSM1229, COSM21978, COSM1233, COSM1232, COSM30551, COSM28637, COSM1235, COSM1234, COSM1239, COSM1238, COSM29015, COSM1241, COSM18896, COSM21976, COSM1243, COSM1245, COSM27069, COSM1251, COSM1247, COSM1248, COSM1250, COSM1249, COSM1255, COSM1252, COSM1253, COSM1254, COSM1256, COSM36293, COSM1257, COSM1260, COSM1333, COSM1258, COSM1264, COSM1265, COSM17946, COSM29442, COSM1270, COSM23560, COSM1273, COSM1275, COSM19029, COSM1277, COSM1334, COSM1285, COSM133754, COSM96888, COSM96883, COSM33966, COSM1289, COSM1290, COSM1293, COSM1294, COSM36305, COSM36313, COSM1297, COSM1299
		13	COSM1304, COSM25064, COSM12706
		17	COSM27910, COSM1310, COSM1311, COSM21979, COSM1312, COSM12711, COSM1314, COSM19285, COSM1315, COSM12710, COSM22379, COSM1317, COSM1316, COSM12709, COSM19109, COSM1321, COSM1322, COSM18681, COSM18682, COSM19110, COSM1323
5	EGFR	18	COSM41905, COSM28508, COSM28511, COSM12988, COSM12371, COSM13009, COSM13427, COSM41603, COSM28601, COSM18441, COSM6252, COSM6253, COSM18425, COSM6239, COSM12373, COSM22992, COSM28510, COSM13979

		19	COSM13432, COSM53194, COSM13181, COSM13182, COSM27041, COSM17570, COSM26509, COSM26038, COSM28517, COSM6223, COSM13184, COSM6225, COSM12728, COSM133189, COSM12678, COSM12386, COSM12367, COSM12384, COSM23571, COSM12419, COSM6220, COSM24267, COSM6218, COSM12382, COSM12383, COSM6254, COSM6255, COSM133197, COSM12387, COSM26704, COSM6210, COSM12369, COSM12370, COSM13185, COSM133207, COSM96856, COSM13556, COSM29274, COSM6256, COSM6268, COSM85993, COSM12423
		20	COSM26445, COSM6241, COSM6242, COSM12376, COSM14068, COSM12427, COSM12378, COSM13428, COSM13005, COSM13433, COSM12377, COSM13006, COSM22954, COSM6226, COSM22940, COSM13007, COSM28513, COSM13189, COSM27110, COSM6240, COSM13190, COSM22951, COSM20891, COSM27568, COSM133565, COSM5945664
		21	COSM12366, COSM26129, COSM6224, COSM12429, COSM12675, COSM12374, COSM6213, COSM14070, COSM13197, COSM28607, COSM53292, COSM33725, COSM28605, COSM13008, COSM13199, COSM26438
6	MET	2	COSM706
		14	COSM707
		16	COSM696, COSM698, COSM703, COSM702, COSM697, COSM701
		19	COSM699, COSM700, COSM691
7	BRAF	15	COSM1138, COSM1137, COSM1136, COSM1135, COSM21542, COSM1134, COSM6267, COSM33729, COSM1132, COSM6265, COSM478, COSM1133, COSM475, COSM477, COSM476, COSM6137, COSM18443, COSM249889, COSM473, COSM474, COSM1130, COSM33808, COSM219798, COSM144982, COSM1128, COSM30730, COSM472, COSM26625, COSM21549, COSM1124, COSM471, COSM1125, COSM1126, COSM470, COSM26506, COSM469, COSM468, COSM1123, COSM467, COSM466, COSM27639
8	KRAS	2	COSM543, COSM12703, COSM20818, COSM542, COSM538, COSM12722, COSM219781, COSM535, COSM536, COSM537, COSM12721, COSM531, COSM87280, COSM532, COSM533, COSM534, COSM527, COSM528, COSM529, COSM12655, COSM523, COSM524, COSM14209, COSM515, COSM519, COSM522, COSM520, COSM521, COSM25081, COSM34144, COSM512, COSM514, COSM516, COSM517, COSM518, COSM87301, COSM511, COSM510, COSM12654, COSM507
		3	COSM554, COSM555, COSM551, COSM552, COSM553, COSM549, COSM550, COSM87298, COSM28518, COSM547, COSM546, COSM87288, COSM1667043
		4	COSM19900, COSM19404, COSM19905
9	AKT1	2	COSM33765
10	HER2	19	COSM683, COSM5029269, COSM14060, COSM51317, COSM13170
		20	COSM20959, COSM12558, COSM303938, COSM12552, COSM12553, COSM18498, COSM18609, COSM14062, COSM26681, COSM303948
		21	COSM14065
11	ALK	Fusion mutation	COSF463, COSF412, COSF734, COSF465, COSF1376, COSF1063, COSF731, COSF480, COSF1543, COSF491, COSF1366, COSF1367, COSF733, COSF1064, COSF1065, COSF1297, COSF475, COSF413, COSF1545, COSF1542, COSF1540
12	ROS1	Fusion mutation	COSF1201, COSF1295, COSF1251, COSF1203, COSF1266, COSF1279, COSF1260, COSF1197, COSF1268, COSF1270, COSF1274, COSF1198, COSF1261, COSF1672, COSF1280
13	RET	Fusion mutation	COSF1272, COSF1492, COSF1233, COSF1512, COSF1610, COSF1254, COSF1235, COSF1263, COSF1256, COSF1242, COSF1504, COSF1510, COSF1514, COSF1482, COSF1341

Appendix Table 3. DNA Positive Control information

Gene	Base Mutation	Amino Acid Mutation	COSMIC ID	Mutation Type
EGFR	c.2573T>G	p.L858R	COSMIC6224	Deletion mutation
KRAS	c.35G>A	p.G12D	COSMIC521	Point mutation
BRAF	c.1799T>A	p.V600E	COSMIC476	Point mutation
HER2	c.2324_2325ins12	p.A775_G776insYVMA	COSMIC20959	Insertion mutation

Appendix Table 4. RNA Positive Control information

Gene	Fusion mutation	COSF ID	Mutation Type
ALK	EML4-ALK.E13:A20.	COSF463	Fusion mutation
ROS1	SLC34A2-ROS1.S4:R32.	COSF1197	Fusion mutation

Appendix Table 5. Information of UDI Recognition Sequences based on Illumina Tech

Strip Color	Number	i7 Sequence	i5 Sequence （NovaSeq, MiSeq）	i5 Sequence （iSeq, MiniSeq, NextSeq）
Purple	UDI-1	TGCATAGC	TAGGATTC	GAATCCTA

	UDI-2	TCTATGCA	GTCGTTGC	GCAACGAC
	UDI-3	GTACGCAT	CCTCGCAT	ATGCGAGG
	UDI-4	AGGTCCTG	AGAAGGCG	CGCCTTCT
	UDI-5	CATGAGCT	ACGTCAGA	TCTGACGT
	UDI-6	AACTCTAG	CATCTGAT	ATCAGATG
	UDI-7	CCGGATGC	GTATCACG	CGTGATAC
	UDI-8	GTACGATA	TGCAACTA	TAGTTGCA
Green	UDI-9	ATTCGATA	ATGGATCG	CGATCCAT
	UDI-10	CGTAGTAC	GCTGAATG	CATTCAGC
	UDI-11	GAGTACGT	CAACTGGC	GCCAGTTG
	UDI-12	TCAGTGCG	TGCAGCAT	ATGCTGCA
	UDI-13	CACACAGT	ACGACCAA	TTGGTCGT
	UDI-14	GTGCATCG	CATTCGGC	GCCGAATG
	UDI-15	TGCGTCAC	GTATGATT	AATCATAC
	UDI-16	ACATCGTA	TGCCTTCA	TGAAGGCA
White	UDI-17	CGGAACGA	GCTGGCTT	AAGCCAGC
	UDI-18	CCTGGCAC	ATAGAGAC	GTCTCTAT
	UDI-19	ATATCGCT	CACATTGA	TCAATGTG
	UDI-20	GACAGTTG	TGGTCACG	CGTGACCA
	UDI-21	TGCCTATG	ACCTTCGG	CCGAAGGT
	UDI-22	GTACCAGT	CGACCATC	GATGGTCG
	UDI-23	AATGTGCA	TAGCATCA	TGATGCTA
	UDI-24	TCGTATAC	GTTAGGAT	ATCCTAAC
Yellow	UDI-25	CTGTGTGT	CGTCGTCT	AGACGACG
	UDI-26	ACAGCACT	ATCCTAGC	GCTAGGAT
	UDI-27	TATCAGTG	GAAGCCTG	CAGGCTTC
	UDI-28	CGGTGTTA	TCGAAGTA	TACTTCGA
	UDI-29	GTCATCAC	ACCGGTAC	GTACCGGT
	UDI-30	GATGTCAG	CATTCAAT	ATTGAATG
	UDI-31	TCACAGCA	TGGTAGCA	TGCTACCA
	UDI-32	AGCACAGC	GTAATCGG	CCGATTAC

Appendix Table 6. Information of UDI Recognition Sequences based on MGI Tech

Strip Color	Number	Sequence
Purple	UDI-1	TAGGATTCTGCATAGC
	UDI-2	GTCGTTGCTCTATGCA
	UDI-3	CCTCGCATGTACGCAT
	UDI-4	AGAAGGCGAGGTCCTG
	UDI-5	ACGTCAGACATGAGCT
	UDI-6	CATCTGATAACTCTAG
	UDI-7	GTATCACGCCGGATGC
	UDI-8	TGCAACTAGTACGATA
Green	UDI-9	ATGGATCGATTCGATA
	UDI-10	GCTGAATGCGTAGTAC
	UDI-11	CAACTGGCGAGTACGT
	UDI-12	TGCAGCATTCAGTGCG
	UDI-13	ACGACCAACACACAGT
	UDI-14	CATTCGGCGTGCATCG
	UDI-15	GTATGATTTGCGTCAC
	UDI-16	TGCCTTCAACATCGTA

Strip Color	Number	Sequence
White	UDI-17	GCTGGCTTCGGAACGA
	UDI-18	ATAGAGACCCTGGCAC
	UDI-19	CACATTGAATATCGCT
	UDI-20	TGGTCACGGACAGTTG
	UDI-21	ACCTTCGGTGCCTATG
	UDI-22	CGACCATCGTACCAGT
	UDI-23	TAGCATCAAATGTGCA
	UDI-24	GTTAGGATTCGTATAC
Yellow	UDI-25	CGTCGTCTCTGTGTGT
	UDI-26	ATCCTAGCACAGCACT
	UDI-27	GAAGCCTGTATCAGTG
	UDI-28	TCGAAGTACGGTGTTA
	UDI-29	ACCGGTACGTCATCAC
	UDI-30	CATTCAATGATGTCAG
	UDI-31	TGGTAGCATCACAGCA
	UDI-32	GTAATCGGAGCACAGC