RingCap®

Human Ovarian Cancer Gene Mutations Detection Kit

High-Throughput Sequencing

Instruction for Use

Product Name

Human Ovarian Cancer 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 whole exon and exon-intron junctions in TP53, BRCA1, BRCA2, KRAS, NRAS, BRAF, PIK3CA, CTNNB1 and PTEN with FFPE pathological tissue from patients with ovarian cancer, as well as the amplification state of the HER2, EMSY and CCNE1. It can also be used to detect the mutation state of genes such as TP53, BRCA1, BRCA2 and PTEN in peripheral blood samples. The test results are only used for scientific research projects, not for clinical diagnosis.

Ovarian cancer is one of the common gynecological tumors. The annual incidence rate ranks third among female reproductive system tumors, behind cervical cancer and endometrial cancer, while the fatality rate ranks first among female reproductive tract malignant tumors. About 80% of ovarian cancers belong to epithelial ovarian cancer, and high-grade serous ovarian cancer (HGSOC) accounts for 70%-80% of ovarian epithelial cancer. The driving changes of high-grade serous ovarian cancer mainly include TP53 mutation, breast cancer susceptibility gene BRCA1 mutation, BRCA2 mutation and cyclin E1 (CCNE1) and EMSY gene amplification. Among them, the rate of TP53 mutation is the highest. KRAS, NRAS and BRAF gene mutations exist in low-grade serous ovarian cancer, CTNNB1 mutations exist in endometrial ovarian cancer, PIK3CA mutations exist in clear cell ovarian cancer, and HER2 amplification is associated with poor prognosis of ovarian cancer.

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 the different sequencing principles. NGS enables the sequencing of up to millions of target nucleic acids at once, provides abundant variation information in a short time and at a relatively low cost. Highlighting the characteristics of high output and high rebuffer, NGS has drawn more and more attention in multiple signaling pathways and targets studies of cancer.

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, and RingCap® mediated amplification allows terminal modification of the products with specific sequences. With the combination of a particular PCR program and Ring-Cap® enzyme, 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	**ILL-OC enriching PCR strip 1**	Primer, dNTPs, Mg^{2+}, PCR solution	Blue	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.
2	**ILL-OC enriching PCR strip 2**	Primer, dNTPs, Mg^{2+}, PCR solution	Pink	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.
3	**UDI 1-8 ligation reaction strip**	UDI, dNTPs, Mg^{2+}, PCR solution	Purple	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each Tube Represents an UDI number
4	**UDI 9-16 ligation reaction strip**	UDI, dNTPs, Mg^{2+}, PCR solution	Green	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each Tube Represents an UDI number

5	**UDI 17-24 ligation reaction strip**	UDI, dNTPs, Mg^{2+}, PCR solution	White	——	——	——	20 μL	8 tubes	1 strip	Each Tube Represents an UDI number
6	**UDI 25-32 ligation reaction strip**	UDI, dNTPs, Mg^{2+}, PCR solution	Yellow	——	——	——	20 μL	8 tubes	1 strip	Each Tube Represents an UDI number
7	**RingCap-Taq (1#)**	Taq enzyme	——_	15 μL	1 tube	——	15 μL	2 tubes	——	——
8	**OC Negative Control**	Purified water	——_	500 μL	1 tube	——	500 μL	1 tube	——	——
9	**OC Positive Control**	Wild-type DNA	——_	20 μL	1 tube	——	20 μL	2 tubes	——	——

Note: In UDI ligation reaction strips, different UDI numbers respectively contain 32 different IlllDx recognition sequences (see Appendix Table 1); The reaction buffer has been pre-loaded in the 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).

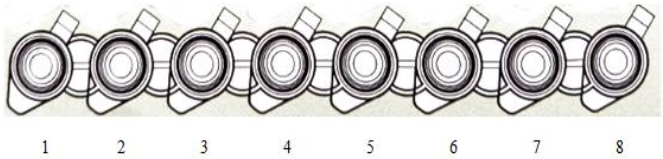

Figure 1. UDI numbers of 8-tube strips

Note: The components of different batches of reagents cannot be mixed.

Equipment and Reagents Required

1. Fluorometer: Promega Quantus™ Fluorometer, Cat. No. E6150; Thermo Fisher Scientific Qubit® 4.0 Fluorescence Meter, Cat. No. Q32866;
2. Nucleic acid extraction kit: Xiamen Spacegen Co., Ltd Nucleic acid extraction (FFPE DNA Magnetic beads) kit Cat. No. SPG-HSD002; Xiamen Spacegen Co., Ltd Nucleic acid extraction (Peripheral blood DNA Centrifugal column method) kit Cat. No. SPG-HSB001;
3. Quantification kit of nucleic acids: Promega, QuantiFluor® dsDNA System, Cat. No. E2670; Thermo Fisher Scientific Qubit® dsDNA HS Assay Kit, Cat. No. Q32851/Q32854;
4. Magnetic beads: Xiamen Spacegen Co., Ltd SGpure beads, Cat. No. SPG-PB001; Magnetic beads Kit from Beckman Coulter, product number A63880/A63881/A63882;
5. Sequencing reagents: Selecting the corresponding sequencing reagent according to the gene sequencer;
6. Illumina PhiX Control v3 (Illumina), Cat. No. FC-110-3002;
7. TE buffer (pH 8.0);
8. Absolute ethanol (Analytical Grade);
9. Nuclease-free water;
10. Nuclease-free tips.

Storage and Stability

1. Storage Condition: Store the kit away from light at - 20±5°C, valid for 9 months, and is not influenced by bottle openings, moreover, do not use the reagents after 5 repeated freeze-thaw cycles.
2. Transportation Condition: The kit should be transported in foam cases with ice bags, with transporting time of less than one week and transporting temperature lowers than 25°C.
3. Check the labels for the production date and expiration date of the kit.

Applicable Instruments

1. PCR system/ thermal cycler: ABI 9700, ABI 2720, ABI Veriti, ABI MiniAmp;

2. Sequencing instruments: Illumina Sequencer.

Specimen Material

The quality of the DNA is critical. Therefore, collecting samples according to the sample types and requirements, followed by DNA extraction:

1. Recommended sample types: FFPE pathological tissue or slices, peripheral blood;
2. FFPE samples: ensure that at least 20% of the collected pathological tissue is tumor lesions; choose FFPE samples that have not been stored for more than 2 years; extract DNA with at least 8 slices of 5 μm section or at least 5 slices of 10 μm section;
3. Peripheral blood: Peripheral blood should be collected with a non-heparin anticoagulant with volumes no less than 2 mL;
4. Commercial kits are highly recommended to extract genomic DNA from the samples. Assess the quality of sample DNA with an ultraviolet-visible spectrophotometer (UV–vis), the ratio of OD_{260}/OD_{280} should be within the range of 1.8-2.2; Quantify sample DNA with a fluorescence meter, the concentration should be ≥ 5 ng/μL, total amount of DNA should be ≥ 50 ng. Once the DNA quantity or quality did not conform to the above requirements, re-extract DNA with a resample or a larger amount of samples. Proceed to library construction after DNA extraction or store at -20±5°C for less than 12 months.

Experimental Procedure

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

I. Library Enrichment

1. Reagent preparation: unfreeze the **ILL-OC enriching PCR strip 1 (blue)** and **ILL-OC enriching PCR strip 2 (pink)** at room temperature according to the total number of samples, briefly centrifuge the tubes before use; place the **RingCap-Taq (1#)** on ice after centrifugation;
2. Sample preparation: According to the effective DNA concentration measured by the fluorometer, dilute the sample DNA to 5 ng/μL, and the volume is ≥ 10 μL, which is the sample DNA to be tested;
3. Enriching PCR reaction:
   a) Pipet 0.5 μL RingCap-Taq (1#) to 10 μL of the "DNA Sample", "OC-PC", and "OC-NTC", vortex slightly followed by brief centrifugation;
   b) Gently remove the cap of enriching PCR strip, for one sample, respectively pipet 5 μL into ILL-OC enriching PCR tube 1 (blue) and ILL-OC enriching PCR tube 2 (pink), replace the cap carefully;
   c) Centrifuge the tubes/ strips slightly to dislodge bubbles;
4. Load the PCR reaction tubes/ strips into the thermal cycler, then run the following program:

Table 2. PCR amplification procedure

Step	Temperature	Time	Cyclic number
Predenaturation	98 °C	2 minutes	1
Denaturation	98 °C	15 seconds	15
Annealing	65 °C	4 minutes	
Storage	4 °C	∞	1

Note: Proceed to "Purification of Enriching Products", or store the products at 2 ~ 8°C within 8 hours or at -20±5°C within 24 hours. Storing for more than 24 hours is not suggested.

II. Purification of Enriching Products

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

1. Mix the two PCR enrichment products of one sample into a new 1.5 mL Eppendorf tube, add 50 μL magnetic beads to each tube, pipet up and down to mix the bead suspension thoroughly with the product;
2. Incubate the mixture for 5 minutes at room temperature;

3. Place the tube on a magnet rack, then incubate for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
4. Pipet 150 µL of freshly prepared 70% ethanol into each tube, rotate the tube side-to-side in the two positions of the magnetic rack 5 times to wash magnetic beads, place the tube on the magnetic rack for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
5. Repeat step 4 for a second wash;
6. Remove all the ethanol from the tube, and keep the tube on a magnetic rack for 5 minutes to air-dry 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, and vortex thoroughly (alternatively, mix by pipetting at least half the total volume up and down), briefly centrifuge to collect the droplets, Incubate the mixture for 5 minutes at room temperature;
8. Place the tube on a magnetic rack for 2 minutes until the solution is clear, carefully remove and store the supernatant (i.e. **purified product**) at - 20±5°C or proceed to "Library Construction".

III. Library Construction

1. Reagent preparation: unfreeze the **UDI ligation reaction strip** at room temperature, briefly centrifuge the tubes before use; place the **RingCap-Taq (1#)** on ice after centrifugation;
2. OC library construction:
   a) Pipet 0.25 µL RingCap-Taq (1#) to 5 µL of the purified products of "DNA Sample", "OC-PC", and "OC-NTC", vortex slightly followed by brief centrifugation;
   b) Gently remove the cap of the UDI ligation strip, sequentially pipet 5 µL of the template prepared above into the respective tube, and replace the cap carefully;
   c) Centrifuge the tubes slightly to dislodge bubbles;
3. Load the PCR reaction tubes/ strips into the thermal cycler; then run the following program:

Table 3. PCR amplification procedure

Step	Temperature	Time	Cyclic number
Predenaturation	98 °C	2 minutes	1
Denaturation	98 °C	15 seconds	25
Annealing	65 °C	4 minutes	
Storage	4 °C	∞	1

Note: Proceed to "Library Purification", or store the products at 2 ~ 8°C within 8 hours or at -20±5°C within 24 hours. Storage for more than 24 hours is not suggested.

IV. Library Purification

Note: Bring 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 the PCR library product to a new 1.5 mL Eppendorf tube, add 25 µL of magnetic beads to each tube, and pipet up and down to mix the bead suspension thoroughly with the product;
2. Incubate the mixture for 5 minutes at room temperature;
3. Place the tube on a magnet rack, then incubate for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
4. Pipet 150 µL of freshly prepared 70% ethanol into each tube, rotate the tube side-to-side in the two positions of the magnetic rack for 5 times to wash magnetic beads, place the tube on a magnetic rack for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
5. Repeat step 4 for a second wash;
6. Remove all the ethanol from the tube, and keep the tube on a magnetic rack for 5 minutes to air-dry magnetic beads (avoid over-dry);

7. Remove the tube from magnetic rack, add 35 μL of TE buffer (pH 8.0) to each tube, and vortex thoroughly (alternatively, mix by pipetting at least half the total volume up and down), briefly centrifuge to collect the droplets; Incubate the mixture for 5 minutes at room temperature
8. Place the tube on a magnetic rack for 2 minutes until the solution is clear, carefully remove and store the supernatant (i.e. **library**) at -20±5°C 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 the OC-NTC library, cannot fragments shall be detected at larger than 200 bp; for OC-PC and all sample libraries, the main fragments should be at 250 ~ 350 bp; for library effective concentration lowers than 1 ng/μL (measured by fluorometer), either of which is decided unqualified;
2. The concentration of Phix Control V3 more than 5% (e.g.the percentage of Phix Control V3 shoule be more than 30 μL in the 600 μL loading volume);
3. Sample dilution and denaturation according to the matching Illumina sequencing kit (refer to the operation manual of each piece of equipment);
4. Store undiluted sample libraries at - 20±5°C for up to 7 days; The mixture of diluted libraries is suggested to be used right after it is ready.

VI. Bioinformatics Analysis

Transfer the Fastq files obtained by sequencing to the analysis server, followed perform data quality control, sequence alignment, mutation annotation analysis-based on the clinical NGS sequencing data analysis system (abbreviated as analysis system below) of Xiamen Spacegen Biotechnology Co., Ltd.

Positive Judgment Value

1. Standard quality control: For DNA sample libraries, the main fragments should be at 250 ~ 350 bp, Uniformity ≥ 75%, mean Depth ≥ 500×;
2. Mutated positive judging criteria:
   a) Peripheral blood sample: In the result of germline variation analysis, if effective depth > 100× and mutation frequency > 25%, this mutation site is judged as positive mutation; Otherwise, it is judged as negative.
   b) FFPE: In the result of somatic variation analysis, if effective depth > 100× and mutation frequency > 1%, the site of this mutation is judged as positive mutation; Otherwise, it is judged as negative or below the detection limit.
3. The result of CNV: The total reads of the target gene accounted for the total reads of the target region/the average of the percentage of the target gene with baseline samples, followed have the ratio result. If the ratio ≥ 2, it is judged as positive amplification; Otherwise, it is judged as negative amplification.

Interpretation of Results

1. NTC libraries should not fragments shall be detected at larger than 200 bp; Otherwise, this test is invalidated;
2. For DNA positive control library, the target fragment should be in 250 ~ 350 bp as well as Uniformity should be more than 75%, moreover, Mean Depth more than 500×; Otherwise, this test is invalidated
3. 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:
   a) Clear clinical significance (I): Diagnostic\prognostic marker of specific tumor or drugs recommended\approved in the professional guidelines;
   b) Potential clinical significance (II): Diagnostic\prognostic marker of specific tumor or drugs that has level A evidence of another tumor in the multiple small research;
   c) Unknown clinical significance (III): It is not found higher rates of variants in the general population and tumor databases, moreover, not has clear published evidence;
   d) Harmless or may be harmless clinical significance (IV): It is found higher rates of variants in the general population and not published evidence.
4. The grade of germline variation based on the "Standards and guidelines for the interpretation of sequence variants: a joint consensus

recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology" and another standard of genetic variation, variation types, and conserved biological functional prediction, evidence of databases(e.g.ClinVar、COSMIC) and literature could divide into 5 levels

a) Pathogenic: There is clear evidence that this variant is associated with an increased risk of cancer；
b) Likely pathogenic: There is moderate evidence that this variant is associated with an increased risk of cancer；
c) Uncertain significance: There is unclear evidence that this variant is associated with an increased risk of cancer；
d) Likely benign: There is moderate evidence that this variant is unrelated to an increased risk of cancer；
e) Benign: There is clear evidence that this variant is unrelated to an increased risk of cancer.

Limitation of the Kit

Testing results obtained from the kit should only be taken as a scientific reference. The instruction shall not interpret the results for mutation sites that were not included in the kit or DNA extracted from samples that were not collected according to designated requirements.

Physical Performance of Products

1. The kit should have a neat appearance, clear markings, and no leakage. When unfrozen, the reagents shall be clear, without sediments.
2. The consistency rates positive reference samples are 100%.
3. The consistency rates negative reference samples are 100%.
4. The kit allows the detection of 1% of specific gene mutations in a 25 ng DNA organizational sample.
5. The repeatability is 100% by detecting the designated sample 10 consecutive times, and all tested were corresponding mutation positive.

Precautions and Warning

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. 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, purification of extracted DNA with magnet beads is highly suggested. Purified DNA should be stored as required (-20±5°C) or proceed to further steps immediately;
8. Do not substitute any original reagents contained in the kit. Do not mix reagents of 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 reagents preparation and template addition.
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.

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.

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Appendix Table 1.

Information of 32 IllDx Recognition Sequences Based on Illumina

Number	i7	i5	Number	i7	i5
UDI-1	TGCATAGC	TAGGATTC	UDI-17	CGGAACGA	GCTGGCTT
UDI-2	TCTATGCA	GTCGTTGC	UDI-18	CCTGGCAC	ATAGAGAC
UDI-3	GTACGCAT	CCTCGCAT	UDI-19	ATATCGCT	CACATTGA
UDI-4	AGGTCCTG	AGAAGGCG	UDI-20	GACAGTTG	TGGTCACG
UDI-5	CATGAGCT	ACGTCAGA	UDI-21	TGACCATT	ACCTTCGG
UDI-6	AACTCTAG	CATCTGAT	UDI-22	GTCCTAGG	CGACCATC
UDI-7	CCGGATGC	GTATCACG	UDI-23	AATGTGCA	TAGCATCA
UDI-8	GTACGATA	TGCAACTA	UDI-24	TCGTATAC	GTTAGGAT
UDI-9	ATTCGATA	ATGGATCG	UDI-25	CTGTGTGT	CGTCGTCT
UDI-10	CGTAGTAC	GCTGAATG	UDI-26	ACAGCACT	ATCCTAGC
UDI-11	GAGTACGT	CAACTGGC	UDI-27	TATCAGTG	GAAGCCTG
UDI-12	TCAGTGCG	TGCAGCAT	UDI-28	GGCATTAC	TCGAAGTA
UDI-13	CACACAGT	ACGACCAA	UDI-29	CTGTGCTA	ACCGGTAC
UDI-14	GTGCATCG	CATTCGGC	UDI-30	GATGTCAG	CATTCAAT
UDI-15	TGCGTCAC	GTATGATT	UDI-31	TCACAGCA	TGGTAGCA
UDI-16	ACATCGTA	TGCCTTCA	UDI-32	AGCACAGC	GTAATCGG