RingCap®

Chemotherapy Pharmacogenomics Detection Kit

High-Throughput Sequencing

Instruction for Use

For Research Use Only

Instruction Version: S1.1 Revision Date: October 2023

Product Name

Chemotherapy Pharmacogenomics Detection Kit (High-Throughput Sequencing)

Packing Specification

16 Tests /kit, 32 Tests /kit

Intended Use

The kit is intended for the qualitative detection of single nucleotide polymorphism (SNP) of genes involved in drug metabolism (see Appendix Table 1), with nucleic acids of peripheral blood collected from patients. The results are only for scientific reference.

Changes in expression level and the genetic variation of genes involved in drug metabolism, drug transportation, and drug targets will cause individual differences in drug response by affecting the concentration and sensitivity of drugs in human bodies. Pharmacogenomics has become an important tool to guide clinically individualized medication and the development of new drugs, and evaluate the risk of adverse drug reactions and efficacy of new drugs. The detection of genes encoding drug-metabolizing enzymes and drug targets could guide clinical selection of appropriate drugs and dosages for individual patients, thus to improving the efficacy and safety of drug therapy, and preventing the occurrence of serious adverse drug reactions.

The kit detects 50 SNP sites of 26 genes related to chemotherapeutics were reported mainly from literatures and generally acknowledged in clinical treatment.

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 once, provides abundant variation information in short time and at relatively low cost. Highlighting the characteristics of high throughput 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.

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 regions, RingCap® mediated amplification allows terminal modification of the products with specific sequences. With the combination of particular PCR program and RingCap-Taq enzyme, library construction of target regions could be achieved on common PCR apparatus before they are ready for high-throughput sequencing.

Kit Contents

Table1 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	**HL 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	**UDI 1-8 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Purple	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each tube represents an UDI
3	**UDI 9-16 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Green	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each tube represents an UDI

4	**UDI 17-24 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	White	——	——	——	20 μL	8 tubes	1 strip	Each tube represents an UDI
5	**UDI 25-32 Reaction Strip**	UDI primer, dNTPs, Mg^{2+}, Buffer	Yellow	——	——	——	20 μL	8 tubes	1 strip	Each tube represents an UDI
6	**RingCap-Taq (1#)**	Taq enzyme	——	10 μL	1 tube	——	10 μL	2 tubes	——	——
7	**HL Negative Control**	nuclease-free water	——	1 mL	1 tube	——	1 mL	1 tube	——	——
8	**HL Positive Control**	Wild-type DNA	——	20 μL	1 tube	——	20 μL	1 tube	——	——

Note1: In UDI reaction strips, different UDI numbers respectively contain 32 different UDI recognition sequences (see Appendix Table 2); the reaction solution has been pre-packaged 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).

Note2: The contents of different batches of reagents cannot be mixed.

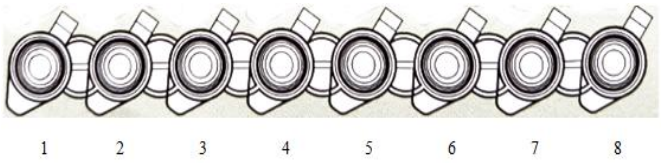

Figure 1 UDI numbers of 8-Tube strips

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: commercial nucleic acids extraction kits are recommended
5. RNA reverses transcription kit: SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher, Cat. No. 11754-050)
6. 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)
7. Magnetic beads: SGpure beads (Xiamen Spacegen Co., Ltd, Cat. No. SPG-PB001) or AMPure XP (Beckman Coulter, Cat. No. A63880/A63881/A63882) or HighPrep™ PCR (MagBio, Cat. No. AC-60005/ AC-60050/ AC-60250/ AC-60500)
8. Sequencing reagents: selecting the corresponding sequencing reagent according to the gene sequencer
9. Illumina PhiX Control V3 (Illumina), Cat. No. FC-110-3002
10. Nuclease-free pipette tips with filter
11. TE buffer (pH 8.0)
12. Nuclease-free water
13. Absolute ethanol (Analytical Grade)

Applicable Instruments

1. Library preparation PCR apparatus: ABI9700, ABI 2720, ABI Veriti, ABI MiniAmp,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 in foam cases with ice bags, with transporting time of less than one week and transporting temperature lower than 25℃.

3. Check labels for the production date and expiration date of the kit.

Specimen Material

The quality of the DNA to be detected is critical. Therefore, collecting samples according to the following recommended sample types, and requirements in clinic, followed DNA extraction:

1. Recommended sample type: peripheral blood.
2. Peripheral blood should be collected with a non-heparin anticoagulant with volumes no less than 2 mL.
3. Extracting DNA from peripheral blood samples store at 2-8°C should not exceed one week.
4. Commercial kit is recommended to extract genomic DNA from 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, the concentration should be ≥ 2 ng/μL, the total amount of DNA should be ≥ 20 ng. Once the DNA quantity or quality was not conformed with the above requirements, re-extract DNA with resampling or a larger amount of sample. Proceed to library enrichment immediately or store DNA sample at -15℃ to -25℃ for no more than 12 months.

Experimental Procedure

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

I. Library Enrichment

1. Reagent preparation: Thaw the **HL PCR Strip** (Blue), HL PC, and HL NTC at room temperature, briefly centrifuge the strip before use. Place the **RingCap-Taq (1#)** on ice after centrifugation.
2. DNA sample: dilute sample DNA 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. Gently remove the cap of the **HL PCR Strip**, add 0.25 μL of the **RingCap-Taq (1#)** into each tube, sequentially add 5 μL sample DNA (2 ng/μL), HL PC and NTC into respective tube, cap the tubes carefully.
4. Centrifuge the tubes slightly and avoid creating air bubbles.
5. Load the PCR strip tubes above into the thermal cycler; then set up and run the program according to Table 2.

Table 2 Library enrichment amplification procedure

Step	Temperature	Time	Cyclic Number
Pre-denaturation	98°C	2 minutes	1
Denaturation	98°C	15 seconds	15
Annealing	65°C	4 minutes	
hold	4°C	∞	1

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

II. Purification of Enriched 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 PCR enrichment product to a new 1.5 mL centrifuge tube, add 25 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 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 150μL of freshly prepared 70% ethanol to each tube, rotate the tubes clockwise and 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 Construction".

III. Library Construction

Note: Use different UDI for different samples/mutations (DNA mutation or RNA fusion mutation).

1. Reagent preparation: Thaw the **UDI Reaction Strip** based on DNA and cDNA 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. Gently remove the cap of the **UDI Reaction Strip**, add 0.25 μL **RingCap-Taq (1#)** into each tube, sequentially add 5 μL purified products of DNA sample, HL PC and HL NTC into 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 Library construction amplification procedure

Step	Temperature	Time	Cyclic Number
Pre-denaturation	98°C	2 minutes	1
Denaturation	98°C	15 seconds	25
Annealing	65°C	4 minutes	
hold	4°C	∞	1

Note: Proceed to "Library Purification", or store the products at 2-8°C 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 a new 1.5 mL centrifuge tube, add 25 μL 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 150μL of freshly prepared 70% ethanol to each tube, rotate the tubes clockwise and 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 HL NTC library, no fragments shall be detected above 200 bp; for HL PC and all sample libraries, the main fragments should be at 200-350 bp; for library effective concentration ≥ 1 ng/μL (measured by fluorometer).
2. The concentration of Phix Control V3 shoule be≥ 5% (e.g.the percentage of Phix Control V3 shoule be ≥ 30 μL in the 600 μL loading volume).
3. Proceed sample dilution and denaturation according to the matching Illumina sequencing kit (refer to operation manual of each equipment).
4. 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

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

VII. Bioinformatics Analysis

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

Data Analysis

Determining results: The outputs of "Variants Report" provide mutation information; check the column "Frequency":

1. For mutation ratio > 85%, that indicates homozygous mutant status of detected sample.
2. For mutation ratio between 25% -85%, that indicates heterozygous mutant status of detected sample.
3. For mutation ratio < 25%, that indicates wild type status of detected sample.

Interpretation of Results

1. HL NTC library should not have any fragment above 200 bp; otherwise, this test is invalidated.
2. For HL PC library, the target fragment should be in 200-350 bp as well as Uniformity should be ≥ 85%, moreover, Mean Depth ≥ 1000×; Otherwise, this test is invalidated.

Limitations of the Kit

1. The results are only scientific references.
2. Situations that may result in false negative or false positive result include but not limit to: Unreasonable sample collection, transportation, improper experimental operations or environment.

Performance characteristics

1. The kit should be neat appearance, clearly labels, and no leakage; when unfrozen, the reagents shall be clear, without sediments or muddy.
2. The consistency rates of positive reference samples shall be 100%.
3. The consistency rates of negative reference samples shall be 100%.
4. The repeatability is 100% by detecting designated sample for 10 repetitive times.

Warnings and Precautions

1. Please read the instruction carefully in prior to experiments.
2. Conduct experiments abiding 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 pre-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 freezing-thawing cycles.
7. The quality of DNA affects experimental results to a great extent, hence, purification of extracted DNA with magnetic beads is highly

suggested. Purified DNA should be stored as required environment (-15℃ to -25℃) 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 which is caused by contamination of reagents.
10. Be cautious of contamination from external DNA; use specific pipettes and tips for reagents preparation and template addition; the place for preparation of the reaction reagents shall be isolated from the place where the temples are added.
11. All reagents in use have potential hazard. For first-use of this kit, the operator may receive training by our technical supports. All used contents of the kit should be considered as clinical dessert and should be disposed properly.

Symbols

Symbol	Symbol definition
	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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7. https://www.nccn.org.
8. www.pharmgkb.org/guidelines.
9. www.accessdata.fda.gov/scripts/cder/ob/UDI.cfm.

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Appendix Table 1:

Detected Sites of the Kit

Number	Genotype	rs ID	Number	Genotype	rs ID
1	ALDH1A1	rs6151031	27	ESR1	rs2813543
2	ABCB1	rs1045642	28		rs4870061
3		rs1128503	29		rs9322335
4		rs2032582	30	ESR2	rs10140457
5		rs2229109	31		rs4986938
6	ABCC2	rs12762549	32	GSTP1	rs1695
7	CASP7	rs12415607	33	MTHFR	rs1801131
8		rs2227310	34		rs1801133
9	CBR3	rs1056892	35	MTRR	rs1801394
10		rs8133052	36	NUDT15	rs116855232
11	CDA	rs2072671	37		rs746071566
12		rs60369023	38		rs777311140
13	CEP72	rs924607	39	SLC19A1	rs1051266
14	CYP2D6	rs1058164	40	SLCO1B1	rs4149081
15		rs1065852	41	TPMT	rs1142345
16		rs1135840	42		rs1800460
17		rs16947	43		rs12201199
18		rs5030865	44		rs200591577
19	DPYD	rs3918290	45		rs1800462
20		rs55886062	46	TYMS	rs11280056
21		rs75017182	47	UGT1A1	rs3064744
22		rs67376798	48	UGT1A1 XPC	rs4148323
23	DYNC2H1	rs716274	49		rs2228001
24	ERCC2	rs13181	50	XRCC1	rs25487
25	ERCC1	rs11615			
26	ATIC	rs4673993			

Appendix Table 2:

Information of 32 UDI Recognition Sequences based on Illumina Tech

Strip Color	UDI Number	i7 Sequence	i5 Sequence
Purple	UDI-1	TGCATAGC	TAGGATTC
	UDI-2	TCTATGCA	GTCGTTGC
	UDI-3	GTACGCAT	CCTCGCAT
	UDI-4	AGGTCCTG	AGAAGGCG
	UDI-5	CATGAGCT	ACGTCAGA
	UDI-6	AACTCTAG	CATCTGAT
	UDI-7	CCGGATGC	GTATCACG
	UDI-8	GTACGATA	TGCAACTA
Green	UDI-9	ATTCGATA	ATGGATCG
	UDI-10	CGTAGTAC	GCTGAATG
	UDI-11	GAGTACGT	CAACTGGC
	UDI-12	TCAGTGCG	TGCAGCAT
	UDI-13	CACACAGT	ACGACCAA
	UDI-14	GTGCATCG	CATTCGGC
	UDI-15	TGCGTCAC	GTATGATT
	UDI-16	ACATCGTA	TGCCTTCA

Strip Color	UDI Number	i7 Sequence	i5 Sequence
White	UDI-17	CGGAACGA	GCTGGCTT
	UDI-18	CCTGGCAC	ATAGAGAC
	UDI-19	ATATCGCT	CACATTGA
	UDI-20	GACAGTTG	TGGTCACG
	UDI-21	TGACCATT	ACCTTCGG
	UDI-22	GTCCTAGG	CGACCATC
	UDI-23	AATGTGCA	TAGCATCA
	UDI-24	TCGTATAC	GTTAGGAT
Yellow	UDI-25	CTGTGTGT	CGTCGTCT
	UDI-26	ACAGCACT	ATCCTAGC
	UDI-27	TATCAGTG	GAAGCCTG
	UDI-28	GGCATTAC	TCGAAGTA
	UDI-29	CTGTGCTA	ACCGGTAC
	UDI-30	GATGTCAG	CATTCAAT
	UDI-31	TCACAGCA	TGGTAGCA
	UDI-32	AGCACAGC	GTAATCGG