CleanPlex™ Targeted Library User Guide

For Targeted Amplicon Sequencing on Illumina® Sequencers

For use with:
CleanPlex™ Targeted Library Kit
CleanPlex™ Generic Cancer Panel

Product Number: 816001, 816002, 816003, 916001, 916002, 916003
Revision 1.0

For Research Use Only. Not for use in diagnostic procedures.

Doc ID: 08081620A

ORDERING INFORMATION

Online: www.paragongenomics.com
Phone: 1-415-545-8783 (US)
Email: orders@paragongenomics.com

Product	Product Number	Pack Size (reactions)
CleanPlex™ Targeted Library Kit	816001, 816002, 816003	8/96/384
CleanPlex™ Generic Cancer Panel	916001, 916002, 916003	8/96/384

CleanPlex™ Custom Panel Design Service

Email: paneldesign@paragongenomics.com

TECHNICAL SUPPORT

Phone: 1-650-822-7545 (US)
Email: techsupport@paragongenomics.com

CUSTOMER SERVICE

Phone: 1-415-545-8783 (US)
Email: customerservice@paragongenomics.com

CleanPlex™ Targeted Library Kit

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1. MATERIALS PROVIDED

1.1 CleanPlex™ Targeted Library Kits

Product	Product Number	Kit Contents
CleanPlex™ Targeted Library Kits	816001	16 µl 5X mPCR Mix
		16 µl CP Reagent Buffer
		16 µl CP Digestion Reagent
		16 µl Stop Buffer
		64 µl 5X 2nd PCR Mix
		1000 µl TE Buffer
	816002	192 µl 5X mPCR Mix
		192 µl CP Reagent Buffer
		192 µl CP Digestion Reagent
		192 µl Stop Buffer
		768 µl 5X 2nd PCR Mix
		4 ml TE Buffer
	816003	768 µl 5X mPCR Mix
		768 µl CP Reagent Buffer
		768 µl CP Digestion Reagent
		768 µl Stop Buffer
		3.08 ml 5X 2nd PCR Mix
		16 ml TE Buffer

**Note: No primer pool is included in any CleanPlex™ Targeted Library Kit. For custom primer panel design, please contact paneldesign@paragongenomics.com*

1.2 CleanPlex™ Generic Cancer Panel

Product	Product Number	Conc.	Primer Pools	Kit Contents
CleanPlex™ Generic Cancer Panel	916001	5X	1	16 µl
	916002			192 µl
	916003			768 µl

2. MATERIALS REQUIRED BUT NOT PROVIDED

- Indexed 2nd PCR primers (New England BioLab, NEBNext® Multiplex Oligos for Illumina®, Dual-Indexed Primers Set 1, #E7600S; Single-Indexed Primers #E7335, #E7500, #E7710, #E7730).
- Thermal cycler.
- 96-well plate or thin-wall PCR strip tubes with attached caps, adhesive film for sealing 96-well plate.
- Agencourt AMPure® XP (Beckman Coulter, A63880, or A63881) or other similar Magnetic Beads (hereafter all referred as Magnetic Beads) for DNA purification.
- DynaMag™ -96 Side (Thermo Fisher Scientific, 12331D) or other similar magnet for 96-well plate or 8/12-well strip tubes.
- Absolute ethanol.
- Nuclease-free water.
- Pipettors and low-retention filtered pipette tips.
- Mini-centrifuge, 96-well plate centrifuge.
- (Optional) Qubit® 3.0 Fluorometer and dsDNA HS (high sensitivity) Assay Kit.
- (Optional) Agilent® 2100 BioAnalyzer® Instrument and Agilent® High Sensitivity DNA Kit.

3. COMPATIBLE SEQUENCING INSTRUMENTS

Illumina® HiSeq Series, NextSeq Series, MiSeq, and MiniSeq

4. STORAGE, HANDLING AND USAGE

CleanPlex™ Targeted Library Kits are shipped on dry ice or ice packs, depending on the country of destination. Upon receipt, store the entire kit at -20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, all kit components will retain full activity for at least one year from the date of receipt.

Always ensure that all frozen components are fully thawed and have been vortexed before use. Before the first use of the kit, thaw all frozen components and spin all components briefly to bring all liquid to the bottom of the tubes.

The components containing enzymes (5X mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) do not freeze at -20°C. These components are specially formulated for storage at -20°C to retain their full activity. Please avoid freezing the kit at below -20°C.

Paragon Genomics CleanPlex™ Targeted Library Kit and CleanPlex™ ready-to-use or custom panel are developed, designed and sold exclusively for research purposes and *in vitro* use. Neither the product, nor any individual component, has been tested for use in diagnostics or for drug development, nor is it for administration to humans or animals. Please refer to the MSDS, which is available upon request.

5. PRODUCT OVERVIEW

CleanPlex™ Targeted Library Kit and CleanPlex™ custom or ready-to-use panels are used together to amplify target regions of DNA using multiplex PCR. The amplification products from multiplex PCR (including amplicons and nonspecific products) are purified and treated with CP Digestion Reagent to degrade the primers and nonspecific PCR products. The amplicons are further purified and re-amplified with primers containing indexes to form a DNA library.

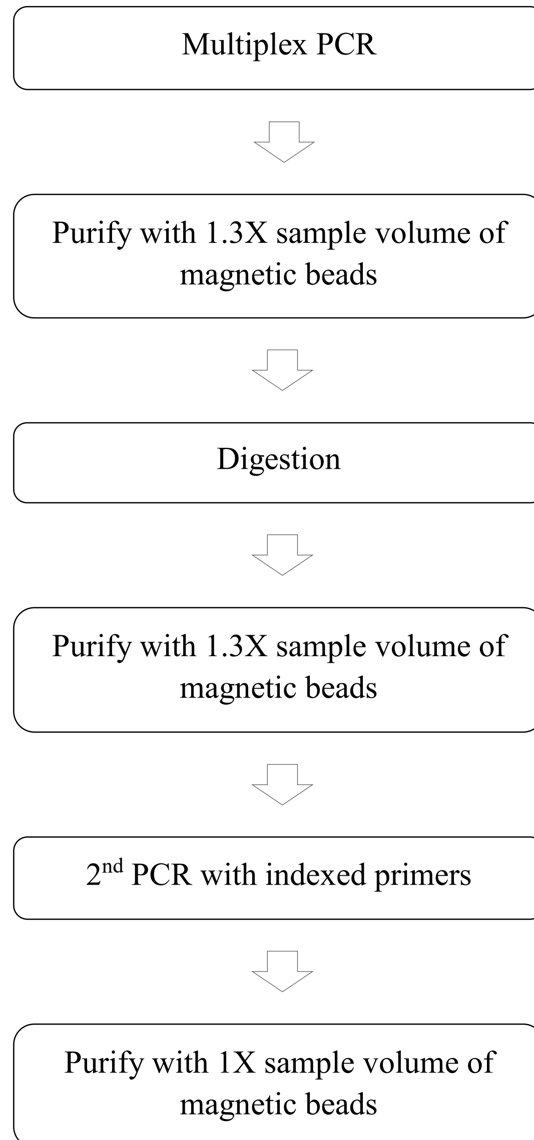
When multiple libraries are made and each library is attached with a unique index, these libraries may be combined (pooled) in a variety of ways before being sequenced on Illumina instruments. Combining libraries maximizes the usage of flow-cell while minimizing cost and labor.

Before sequencing, the final library (either a single library or a pool of libraries each with a unique index) is quantified by qPCR, or by a method compatible with Illumina sequencing instruments.

6. APPLICATIONS

The CleanPlex™ Targeted Library Kit and CleanPlex™ ready-to-use or custom panel are ideally suited for constructing targeted libraries from genomic DNA, DNA from FFPE tissue sections, cell-free DNA (cfDNA), and cDNA derived from RNA by reverse transcription. These targeted libraries are intended for use in Next-Generation Sequencing in detecting nucleotide sequence information related to various cancers, inherited diseases, infections, etc.

7. WORKFLOW



8. PROCEDURE GUIDELINES

For first-time use, briefly spin the tubes of the kit to bring the liquid to the bottom of the tubes. Store the tubes containing enzymes (5X mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) on ice during procedure. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spin-down before use.

Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination. Always change pipette tips between samples.

Use a calibrated PCR thermal cycler as specified in the manufacturer's user guide. Validated thermal cyclers include BioRad C1000, Eppendorf Mastercycler series, Applied Biosystems GeneAmp PCR System 9700. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second up temperature change, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed, or use the default setting (no ramp adjustment).

To ensure accurate assembly of reactions, withdraw viscous solution (such as 5X mPCR mix, CP Digestion Reagent, 5X 2nd PCR mix) from containers slowly and dispense it slowly into reaction mixtures. A good practice is to rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture, followed by mixing the mixture by vigorous vortexing for a few seconds (remember to briefly spin the tubes or 96-well plate afterwards).

9. DETAILED PROTOCOL

9.1 Input DNA Requirements

0.1 – 40ng of human genomic DNA (30-12000 cells) from normal or FFPE tissue, or circulating cell-free DNA (cfDNA) can be used for each multiplex PCR reaction. 10ng of human genomic DNA (3000 cells) is recommended for detection of somatic variants with 1% frequency. When DNA quality is low or unknown (such as DNA from FFPE tissues), higher DNA input usually produces better library quality and lower Limit of Detection. For germline genotyping assays, as low as 0.1ng of high-quality genomic DNA may be used. We recommend the Qubit™ dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) for measuring DNA concentration. UV spectrophotometry methods (e.g. using a NanoDrop™) are not recommended. The maximum volume of template DNA per reaction is 6 µl (for use with 5X concentrated primer pools) or 3 µl (for use with 2X concentrated primer pools).

CleanPlex™ panels are supplied as either 2X or 5X concentrated primer pools. Some CleanPlex™ panels are supplied as multiple primer pools. For these panels, an individual multiplex PCR reaction is required for each primer pool.

Note: *It is a good practice to pre-warm thermal cycles, pre-warm a water bath or heat block to 37°C, and plan assigning indexes or index combinations to specific samples before starting the following steps.*

9.2 Amplify DNA Targets

Step 1. Using thin-wall PCR strip tubes (or 96-well PCR plate for multiple samples), add the components in the following order on ice or a cold block.

Note: *It may be necessary to prepare a master mix excluding DNA samples for multiple reactions. In this case, add DNA samples individually at last to minimize cross-contamination.*

For 5X Primer Pool:	
Nuclease-free water	6 – X µl
5X mPCR Mix	2 µl
5X Primer Pool	2 µl
DNA Sample	X µl
Total:	10 µl

For 2X Primer Pool:	
Nuclease-free water	3 – X µl
5X mPCR Mix	2 µl
2X Primer Pool	5 µl
DNA Sample	X µl
Total:	10 µl

Important! *5X mPCR mix and primer pools are viscous. Pipet slowly and rinse tips when handling these reagents. Mix the master mix by vortexing vigorously for 5 seconds, followed by brief spin.*

Step 2. Close the cap of the strip tubes or seal the 96-well plate with adhesive film, spin briefly to bring down the liquid, mix by vortexing vigorously for 5 seconds, and spin down to collect droplets.

9.3 Thermal Cycling Conditions

Load the tubes or plate in the thermal cycler, and run the following PCR program to amplify target DNA regions.

Step	°C	Time
Pre-heat	95	10 min
10 Cycles	98	15 sec
	60	5/8/16 min*
Hold	10	∞

**5 min for 7-2000, 8 min for 2001-5000, 16 min for 5001-24000 pairs of primers per reaction.*

9.4 Purify the Amplicons

Important! *Bring Magnetic Beads to room temperature and vortex thoroughly to disperse the beads before use. Pipette slowly.* ***Use freshly prepared 70% ethanol****.*

Step 1. Spin the tubes or 96-well plate for 2 seconds. Open the tubes or carefully remove the adhesive film from the 96-well plate. For panels consisting of one primer pool, add 10 µl TE buffer to each sample. For panels consisting of multiple primer pools, combine the 10 µl multiplex PCR reactions for each sample. The total volume of each sample is approximately:

Number of primer pools	Total volume of combined sample
1	(Add 10 µl of TE buffer) 20 µl
2	20 µl
3	30 µl
4	40 µl

Step 2. Vortex Magnetic Beads suspension vigorously for 5 seconds. Add **1.3**X sample volume of Magnetic Beads suspension to each sample. For example, for 20 µl sample volume, add 26 µl of Magnetic Beads suspension to each sample. Close the caps or seal the 96 well plate with adhesive film, vortex vigorously for 5 seconds to thoroughly mix the mixture.

Step 3. Incubate the mixture for 5 minutes at room temperature.

Step 4. Spin the tubes or 96-well plate for 2 seconds. Place the tubes or plate on a magnetic rack and incubate for 2 minutes or until solution clears. The beads will be drawn onto one side of the wall. While keeping the tubes or 96-well plate on the magnet, carefully pipette to remove and discard the supernatant without touching the beads.

Step 5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Take out the tubes or plate from the magnet, place the clear side of the tubes against the magnet to move the beads from one side of the tube to the other side (no need to vortex), then pipette to remove and discard the supernatant without disturbing the beads.

Step 6. Repeat step 4.

Step 7. After the second wash, carefully pipette to remove as much as possible the residual droplets left behind in the tubes (it may require brief spin for 2 seconds to bring down all droplets and magnet the tubes again). Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. **Do not overdry.**

Note: *Residual ethanol inhibits the following reaction. If necessary, briefly spin the tubes or plate. While placing the tubes or plate on magnet, remove residual droplet prior to air-drying the beads.*

Step 8. Add 10 µl TE buffer to each tube. Briefly spin and vortex to resuspend the beads. The DNA will be immediately released from the beads (no need to remove the beads). Brief spin. Go to the next step.

9.5 Digest Nonspecific Products

Note*: Set a water bath to 37°C, or pre warm a thermal cycler to 37°C before the following step.*

Step 1. Open the tubes or carefully remove the 96-well plate seal, add the following reagents to each amplified sample (the beads in the mixture do not affect the reaction).

Add to the Bead Suspension:	
Nuclease-free Water	6 µl
CP Reagent Buffer	2 µl
CP Digestion Reagent	2 µl
Total:	10 µl

Step 2. Close the tubes or seal the plate with adhesive film, vortex thoroughly, and spin down to collect droplets.

Step 3. Incubate at 37°C for 10 minutes (Do **NOT** incubate longer than 10 minutes).

Step 4. Immediately add 2 µl Stop Buffer to each tube, mix by brief spin and vortexing. Spin to bring the liquid to the bottom. The volume of each sample is approximately 22 µl.

9.6 Purify the Amplicons

Step 1. Vortex to resuspend the Magnetic Beads suspension. Add **29 µl** (1.3X sample volume) of Magnetic Beads to each multiplex PCR reaction. The methods described in **Section 9.4 Step 2 to 7** is used again in this step. The only difference is that 29 µl of Magnetic Beads is used. Please refer to Section 9.4 Step 2 to 7 to purify the amplicons.

Step 2. After purification, Add 10 µl TE buffer to each tube. Briefly spin, vortex and spin (no need to remove the beads). Go to the next step.

9.7 Amplify and Add Index

Step 1. Thaw NEB Universal Primer and Index Primers, vortex briefly for 2 seconds, spin briefly for 2 seconds to collect the droplets. Add the following reagents to the above beads-DNA suspension:

Add to the Bead Suspension:	
Nuclease-free Water	18 µl
5X 2nd PCR Mix	8 µl
Total:	26 µl

Note: *It may be necessary to prepare a master mix of above for multiple reactions.*

Then add index primers carefully to the above beads-DNA suspension. Be cautious to minimize cross-contamination of index primers:

Add to Each Specific Sample:	
NEB Universal or i5 Index Primer (10 µM)	2 µl
i7 Index Primer (10 µM)	2 µl

Note: *New England BioLab's NEBNext® Multiplex Oligos include 48 single-indexed primers (#E7335, #E7500, #E7710, #E7730) and Dual-Index Primers (#E7600S) for 96 index combinations. It is necessary to plan beforehand to assign a specific index or index combination to each specific sample.*

Step 2. Close the strip tubes or seal the plate with adhesive film, vortex thoroughly, and spin down to collect droplets.

Step 3. Load the tubes or plate in the thermal cycler, and run the following PCR program.

Step	°C	Time
Pre-heat	95	10 min
Cycle number in chart below	98	15 sec
	60	75 sec
Hold	10	∞

Recommended 2nd PCR Cycle Numbers:

Number of Total Amplicons	10ng of high quality gDNA	10 ng of low quality DNA (cfDNA or FFPE DNA)
7 - 100	13	14
101 - 200	11	12
201 - 500	10	11
501 - 1000	9	10
1001 - 2000	8	9
2001 - 5000	7	8
5001 - 12000	6	7
12001 - 24000	5	6

Note: *The optimal PCR cycler number may need fine-tuning for each specific primer panel. Generally, add 3 cycles for 1 ng of DNA, 6 cycles for 0.1 ng of DNA; reduce 1 cycle for 20 ng of DNA, 2 cycles for 40 ng of DNA.*

Stop point*: PCR products may be stored at 10°C overnight. For longer periods, store at –20°C.*

9.8 Purify the Amplicons

Step 1. Vortex to resuspend the Magnetic Beads suspension. Add **40 µl** (1X sample volume) of Magnetic Beads to each multiplex PCR reaction. The same methods described in **Section 9.4 Step 2 to 7** is used again in this step. The only difference is that 40 µl of Magnetic Beads is used. Please refer to Section 9.4 Step 2 to 7 to purify the amplicons.

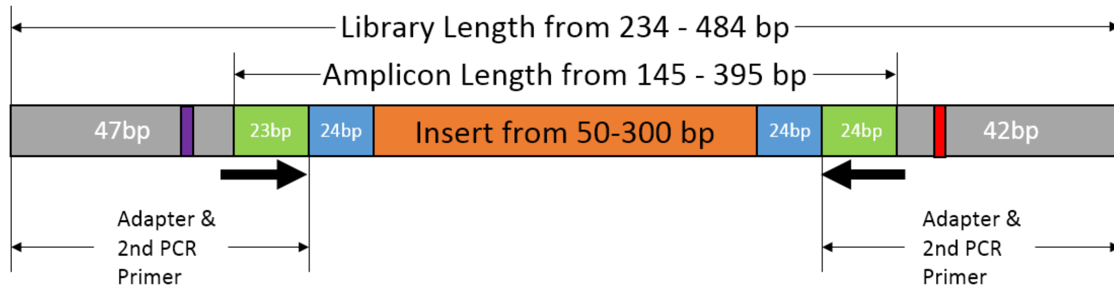
Step 2. Add 10 µl TE buffer to each tube. Briefly spin and vortex to resuspend the beads to elute DNA from beads. Spin briefly to bring the liquid to the bottom. The library is finished.

The library can be stored with beads at -20°C (remember to magnet the beads behind when taking library out for QC and sequencing).

Step 3. (Optional) Measure the concentration of the library by Qubit® 3.0 Fluorometer and dsDNA HS (high sensitivity) Assay Kit. Check library quality and concentration with Agilent® 2100 BioAnalyzer® Instrument and Agilent® High Sensitivity DNA Kit.

10. APPENDIX

10.1 Library Structure (Dual-Indexed)

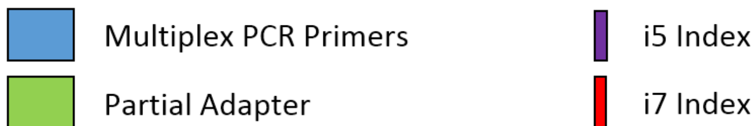


2nd PCR primers (Partial Adapter in Green):

5'AATGATACGGCGACCACCGAGATCTACAC-(i5 Index)-ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

5'CAAGCAGAAGACGGCATACGAGAT-(i7 Index)-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T

Sequencing Primer



10.2 Single-Indexed Primers

Universal Primer:

5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

i7 Index Primer (position of index in red, please refer to New England BioLab's NEBNext® Multiplex Oligos #E7335, #E7500, #E7710, #E7730 for index bases):

5'CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T

10.3 Dual-Indexed Primers

i5 Index Primer (position of index in red please refer to New England BioLab's NEBNext® Multiplex Oligos #E7600S for index bases):

5'AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATC*T

i7 Index Primer (position of index in red please refer to New England BioLab's NEBNext® Multiplex Oligos #E7600S for index bases):
5'CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGC TCTTCCGATC*T

10.4 Troubleshooting

Please visit our web site www.paragongenomics.com/company/frequently-asked-questions for troubleshooting.

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