Takara Bio USA

ThruPLEX® DNA-Seq HV PLUS User Manual

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(020821)

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I. Introduction

A. Overview

ThruPLEX DNA-Seq HV PLUS is designed to provide up to 96 indexed libraries for higher multiplexing capabilities on Illumina® NGS platforms. ThruPLEX DNA-Seq HV PLUS chemistry is engineered and optimized to generate DNA libraries with high molecular complexity and balanced GC representation from input volumes of up to 30 µl. Inputs of 5 ng to 200 ng double-stranded DNA (dsDNA) are required for library preparation. The entire three-step workflow takes place in a single tube or well, in about two hours (Figure 1). No intermediate purification steps or sample transfers are necessary, thus preventing handling errors and loss of valuable samples. With high library diversity, ThruPLEX DNA-Seq HV PLUS libraries excel when combined with target enrichment to deliver high-quality sequencing results.

The ThruPLEX DNA-Seq HV PLUS Kit combines the ThruPLEX HV PLUS Enzymatic Fragmentation Module (Cat. No. R400780, R400781) and ThruPLEX DNA-Seq HV (Cat. No. R400741, R400740). The ThruPLEX HV PLUS Enzymatic Fragmentation Module is designed to perform size-tunable enzymatic fragmentation in tandem with the ThruPLEX DNA-Seq HV repair step. This eliminates additional time for fragmentation, such as mechanical fragmentation, or separate enzymatic fragmentation modules.

Pairing ThruPLEX DNA-Seq HV PLUS with ThruPLEX HV unique dual indexes (UDIs) adds the capability of multiplexing up to 96 NGS-ready libraries. Once purified and quantified, the resulting libraries are ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols. The kit provides excellent results for high-coverage deep sequencing, such as de novo sequencing, whole genome resequencing, whole exome sequencing, and other enrichment techniques.

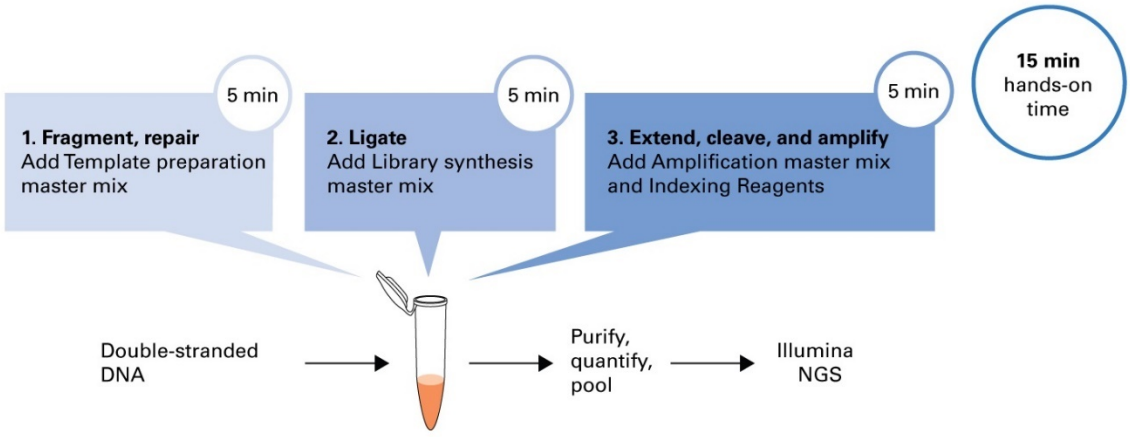

Figure 1. ThruPLEX DNA-Seq HV PLUS single-tube library preparation workflow. The ThruPLEX DNA-Seq HV PLUS workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify and transfer the sample material.

B. Principle

ThruPLEX DNA-Seq HV PLUS is based on our patented ThruPLEX technology (Figure 2). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, ThruPLEX HV uses stem-loop adapters to construct high-quality libraries in a fast and efficient workflow. In the first step, template preparation, the DNA is fragmented and repaired as blunt ends. In the next step, stem-loop adapters with blocked 5' ends are ligated with high efficiency to the 5' end of the genomic DNA, leaving a nick at the 3' end. The adapters cannot ligate to each other and do not have single-stranded tails, preventing any nonspecific background typically found with many other NGS preparations. In the final step, the 3' ends

of genomic DNA are extended to complete library synthesis, and Illumina-compatible indexes are added through high-fidelity amplification. Any remaining free adapters are destroyed. Hands-on time and risk of contamination are minimized by using a single tube and eliminating intermediate purifications.

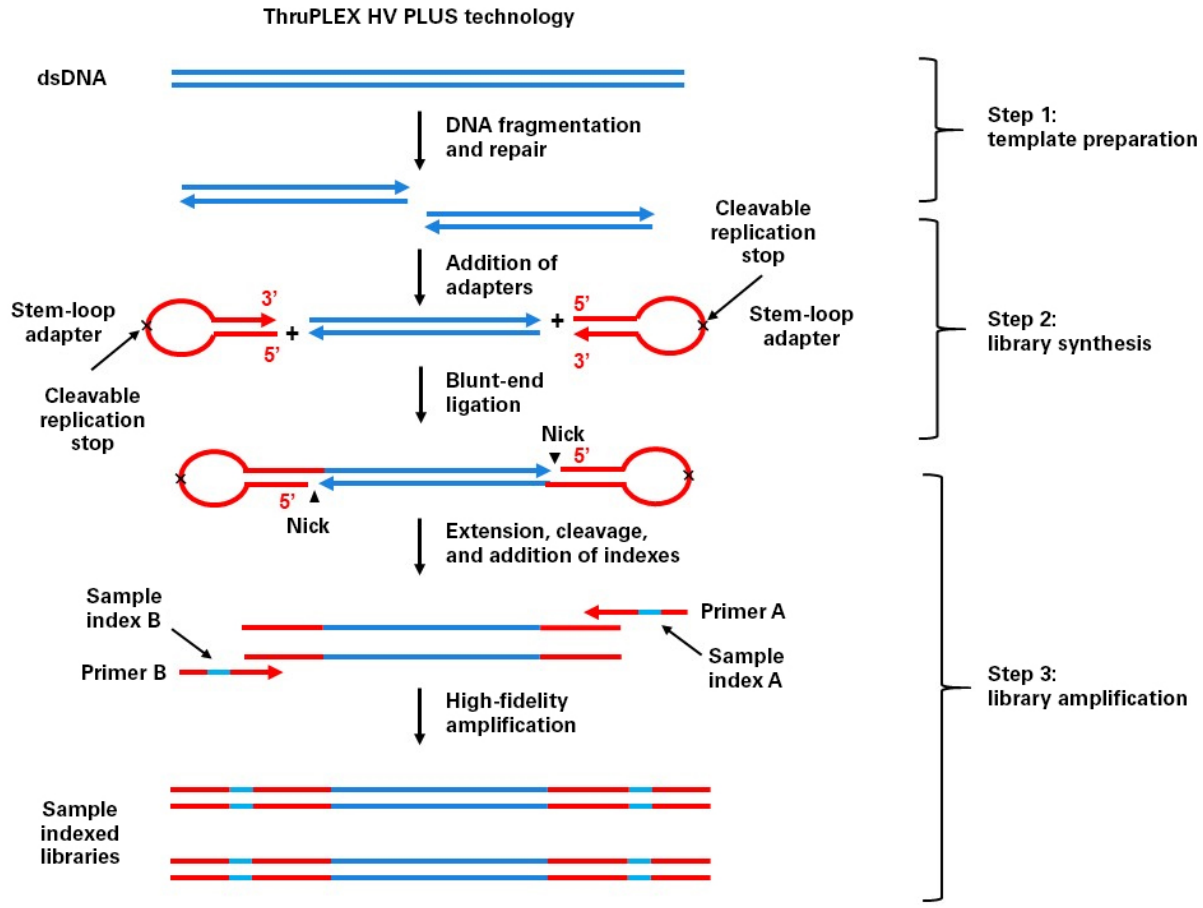

Figure 2. ThruPLEX HV PLUS technology uses a three-step, single-tube reaction that starts with double-stranded DNA (5 ng to 200 ng). Stem-loop adapters are blunt-end ligated to repaired input DNA. These molecules are extended, then amplified to include barcodes using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX DNA-Seq HV PLUS Workflow

The ThruPLEX DNA-Seq HV PLUS workflow is highly streamlined and consists of the following three steps:

- **Template preparation** for fragmentation and repair of the double-stranded DNA input.
- **Library synthesis** for ligation of our patented stem-loop adapters.
- **Library amplification** for extension of the template, cleavage of the stem-loop adapters, and amplification of the library. Illumina-compatible indexes are also introduced using a high-fidelity, highly processive, low-bias DNA polymerase.

The three-step ThruPLEX DNA-Seq HV PLUS workflow takes place in a single tube or well and is completed in about two hours (Figure 3).

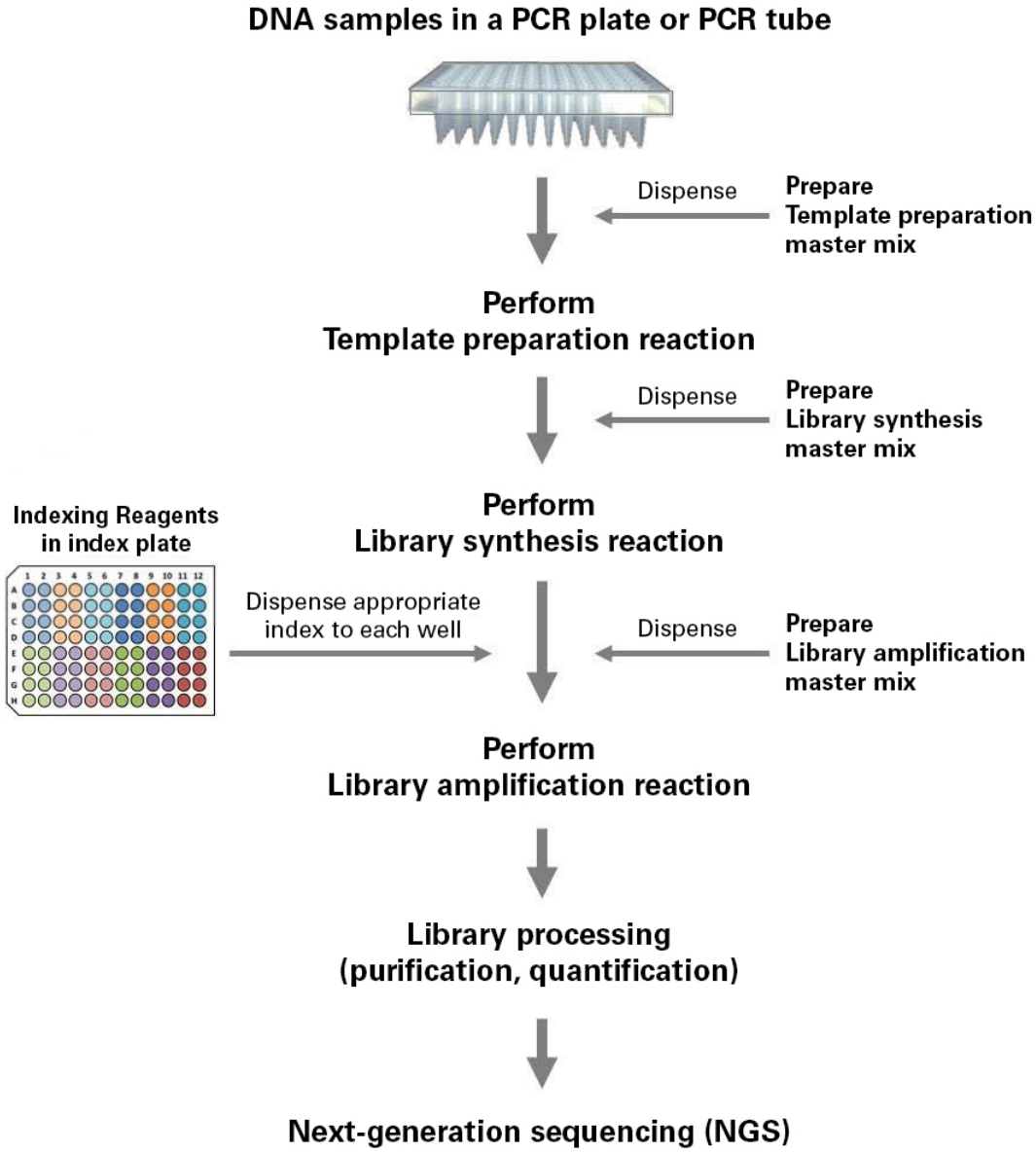

Figure 3. Overview of ThruPLEX DNA-Seq HV PLUS library preparation for Illumina NGS.

II. List of Components

A. Components

Table 1. ThruPLEX DNA-Seq HV PLUS Kit contents

ThruPLEX DNA-Seq HV PLUS		R400782	R400783
ThruPLEX HV PLUS Enzymatic Fragmentation Module	Cap color	R400780 (24 rxns)	R400781 (96 rxns)
PEF1	Green	1 tube	1 tube
PBF1	Yellow	1 tube	1 tube
10X PDF1	Light blue	1 tube	1 tube
Control Human gDNA (5 ng/µl)	Red	1 tube	1 tube
Nuclease-Free Water	White	1 tube	4 tube
ThruPLEX DNA-Seq HV	Cap color	R400741 (24 rxns)	R400740 (96 rxns)
PBD1	Blue	1 tube	1 tube
PED1	Blue	1 tube	1 tube
SBD1	White	1 tube	1 tube
SED1	White	1 tube	1 tube
ABD1	Amber tube	1 tube	4 tubes
AED1	Violet	1 tube	1 tube
Control Fragmented Human gDNA (5 ng/µl)	N/A	1 tube	1 tube
Nuclease-Free Water	Clear	1 tube	1 tube
ThruPLEX HV UDI*		1 Dual Index Plate (24 D)	1 Dual Index Plate (96 D)

*included in bundle part numbers R400740 & R400741; also sold separately as R400738 & R400739

B. Shipping and Storage Conditions

ThruPLEX HV PLUS Enzymatic Fragmentation Module, ThruPLEX DNA-Seq HV, and ThruPLEX HV UDI are shipped on dry ice. The kits should be stored at –20°C upon arrival.

C. Additional Materials Required

- Hot-lid PCR thermal cycler (real-time instrument optional)

  NOTE: See Thermal Cycler Considerations in section III.B.1.
- Centrifuge
- PCR tubes or 96-well nuclease-free thin-wall PCR plates

  NOTE: Select appropriate tubes or plates that are compatible with the thermal cyclers and/or real-time thermal cyclers used. Use appropriate caps or sealing films and seal thoroughly to eliminate evaporation during cycling conditions. Evaporation could reduce robustness and reproducibility of the reactions.
- 1.5 ml low adhesion microcentrifuge tubes
- PCR plate seals (if using plates)
- Single-channel pipettes: 10 µl, 20 µl, and 200 µl
- Multi-channel pipettes: 20 µl and 200 µl
- Low-binding filter pipette tips: 10 µl, 20 µl, 200 µl
- Low-binding aerosol barrier tips
- Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0)
- 80% (v/v) ethanol: freshly made for each experiment
- Magnetic separator, such as SMARTer-Seq™ Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
- Fluorometer, such as Thermo Fisher Scientific Qubit, for library quantification
- Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880) or NucleoMag® NGS Clean-up and Size Select (Takara Bio, Cat. Nos. 744970.5, 744970.50, 744970.500)

NOTE: Agencourt AMPure XP beads and NucleoMag NGS Clean-up and Size Select need to come to room temperature before the container is opened. Therefore, we strongly recommend aliquoting the beads upon receipt, then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). Aliquoting is also essential for minimizing the chances of bead contamination.

Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

D. Optional Materials

- qPCR-based library quantification kit for Illumina NGS libraries: Library Quantification Kit (Takara Bio, Cat. No. 638324)
- Agilent Bioanalyzer or TapeStation, for library size distribution

III. General Considerations

A. Sample Requirements

Table 2. DNA sample requirements

DNA sample requirements	
Nucleic acid	Double-stranded DNA
Source	Cells, fresh tissues, frozen tissues, microbes
Input amount	5 ng to 200 ng
Input volume	30 µl
Input buffer	≤10 mM Tris, ≤0.1 mM EDTA

1. General Guidelines

DNA samples must be dsDNA in order to be used with ThruPLEX DNA-Seq HV PLUS. This kit is **not** for use with single-stranded DNA (ssDNA) or RNA.

NOTE: For degraded or previously fragmented samples that do not require additional enzymatic fragmentation, follow the ThruPLEX DNA-Seq HV User Manual, which skips fragmentation. For additional recommendations, please contact Takara Bio Technical Support. The ThruPLEX DNA-Seq HV User Manual can be downloaded at takarabio.com.

2. DNA Isolation

The table below lists recommended kits for isolation of common sample types. For additional recommendations, please contact Takara Bio Technical Support.

Table 3. Recommended DNA purification kits

Sample type	Recommended kit	Cat. Nos.
Mammalian cells and tissues	NucleoSpin® Tissue	740952.10, 740952.50, 740952.250
Mammalian cells and tissues (low input)	NucleoSpin Tissue XS	740901.10, 740901.50, 740901.250

3. Input DNA Amount

The recommended input amount is 5 ng to 200 ng of DNA quantified by Qubit Fluorometer or equivalent methods. Use an appropriate input amount of DNA to ensure sufficient variant copies are available to achieve the desirable detection sensitivity. In general, detection of alleles present at low frequencies requires a higher input amount of DNA.

Table 4. Estimated genome copies based on input amount and allele frequency

Estimated genome copies available for library preparation				
Input amount	Total haploid genome copies*	Total variant copies at indicated allele frequency: 5%	1%	0.5%
100 ng	33,333	1,666	333	166
50 ng	16,666	833	166	83
10 ng	3,333	166	33	16
5 ng	1,666	83	16	8

*Calculated using 3 pg as the mass of a haploid genome. The genomic complexity of samples is highly variable. All numbers are rounded down to the nearest whole number.

4. Input Volume

The maximum sample input volume is 30 µl. If a sample is a larger volume, it must be concentrated to 30 µl or less. Ensure the buffer concentration remains appropriate (see below).

5. Input Buffer

Input DNA must be eluted or resuspended in a low-salt, low-EDTA buffer. The concentrations of Tris and EDTA must not exceed 10 mM and 0.1 mM, respectively. Avoid phosphate-containing buffers. The preferred buffer is low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

6. Fragment Size

ThruPLEX HV PLUS Enzymatic Fragmentation Module provides a protocol optimal for 300- and 450-bp DNA fragment sizes. ThruPLEX DNA-Seq HV PLUS is a ligation-based technology; ligated adapters result in an approximately 140-bp size increase of each DNA template fragment. Library molecules with shorter inserts (200–300 bp) tend to cluster and amplify more efficiently on Illumina flow cells. Depending on the application and requirements, the solid-phase reversible immobilization purification step following **Library amplification** can be replaced with a size-selection step to remove unwanted fragments.

7. Positive and Negative Controls

Include appropriate positive and negative controls in the experimental design to help verify that reactions proceed as expected. If the experimental samples contain any carryover contaminant(s) in the buffer, the downstream reactions may be impacted, and inclusion of controls would help elucidate such problems. Always prepare fresh dilutions of reference DNA (Control Human gDNA, included in the ThruPLEX DNA-Seq HV PLUS Enzymatic Fragmentation Module). Include a negative control (no template control, NTC) with low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) or Nuclease-Free Water. The positive control and experimental samples should perform equivalently, while the NTC should not amplify.

B. General Recommendations

1. Thermal Cycler Considerations

Thermal cycling and heated lid

Use a thermal cycler equipped with a heated lid that can accommodate 100-µl reaction volumes. Set the temperature of the heated lid to 101°C–105°C to avoid sample evaporation during incubation and cycling.

Thermal cycler ramp rates

We recommend a ramp rate of 3°C/s–5°C/s; higher ramp rates are not recommended and could impact the quality of the library.

2. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared fresh at each workflow step, based on the number of reactions to be performed. Prepare ~10% excess of each master mix to allow for pipetting losses.

1. Transfer enzymes onto ice just prior to use and centrifuge briefly to collect contents at the bottom of the tube prior to use.
2. Thaw buffers on ice, vortex briefly, and centrifuge prior to use.
3. Keep all components and master mixes on ice.
4. Once the master mix is prepared, thoroughly mix the contents several times with a pipette, avoiding the introduction of air bubbles. Briefly centrifuge prior to dispensing into the PCR plate or tube(s).

The **Library synthesis master mix** and **Library amplification master mix** can be prepared during the last 15 min of the previous step's cycling protocol and kept on ice until use.

3. Indexing Reagents

We support the use of ThruPLEX HV index kits with ThruPLEX HV PLUS. The indexing modules are available for purchase separately or bundled with the core enzymatic components. They are available in 24- or 96-reaction sizes and as unique dual indexes.

NOTE: The ThruPLEX DNA-Seq HV PLUS Kit is **NOT** compatible with other versions of Takara Bio indexing kits, including those sold with previous versions of ThruPLEX kits. Contact Technical Support with any questions on compatibility.

Indexing Reagents consist of amplification primers containing Illumina-compatible indexes. Index sequences can be downloaded as .xlsx files from the ThruPLEX DNA-Seq HV PLUS Product Page, under the Resources tab. Before starting the ThruPLEX HV PLUS Library Preparation Protocol (section IV.A), refer to Appendix A for information on index sequences, index plate handling instructions, and guidelines for multiplexing and index-pooling.

ThruPLEX HV UDI 24- and 96-reaction kits

Indexing Reagents are pre-dispensed and sealed in a linear barcoded index plate. The index plate is sealed with foil that can be pierced with a multichannel pipette tip to collect the index to assemble the reactions. Each well of the index plate contains sufficient volume for a single use. No more than four freeze-thaw cycles are recommended for the index plate.

4. Using Illumina Experiment Manager

Install the latest version (v1.18.1 or later) of the Illumina Experiment Manager (IEM). Prior to starting the ThruPLEX DNA-Seq HV PLUS Library Preparation Protocol (section IV.A), create a Sample Sheet in the IEM to select and validate appropriate indexes to use in your experiments. Refer to Appendix A for guidelines on using the IEM to validate your index combinations.

5. Target Enrichment

The library prepared using ThruPLEX DNA-Seq HV PLUS is compatible with major target enrichment products. The target enrichment protocols can be accessed through the Learning Center at takarabio.com.

C. Safety Guidelines

Follow standard laboratory safety procedures and wear a suitable lab coat, protective goggles, and disposable gloves to ensure personal safety as well as to limit potential cross contaminations during the sample preparation and subsequent amplification reactions. For more information please refer to the appropriate Safety Data Sheet (SDS) available online at takarabio.com.

IV. Protocols

A. Protocol: ThruPLEX DNA-Seq HV PLUS Library Preparation

1. Template Preparation

In this section, dsDNA templates will be prepared by fragmentation and subsequent repair. ThruPLEX DNA-Seq HV PLUS provides protocols to generate fragmented DNA products of 300 bp and 450 bp. Fragment size is controlled by varying the concentration of the fragmentation enzyme PEF1 (see table in IV.A.1.3). The reaction occurs at room temperature; samples and reaction mixture must be placed on ice to prevent unintended reactions.

Optimization of PEF1 fragmentation enzyme dilution for DNA fragmentation:
The required concentration of PEF1 depends on the amount, quality, and source of input DNA, as well as the thermal cycler used.

- **Optimization experiment:** An optimization experiment to identify the appropriate fragmentation size is recommended. Determine the optimal dilution amount of PEF1 based on the **Enzyme dilution guide** below for the desired amount of input DNA. Use this optimal dilution of PEF1 in the actual experiment.

Enzyme dilution guide

Reagent	Input DNA >50 ng		Input DNA ≤50 ng	
	300 bp	450 bp	300 bp	450 bp
1X PDF1	16 µl	25 µl	20 µl	30 µl
PEF1	2 µl	2 µl	2 µl	2 µl

NOTE: Assemble all reactions in thin-wall, 96-well PCR plates or PCR tube(s) compatible with the thermal cycler and/or real-time thermal cycler to be used.

1. Prepare samples as described below:
   - **Samples:** dispense 30 µl of dsDNA into each PCR tube or well of a PCR plate.
   - **Positive control reactions:** assemble reactions using 30 µl of the provided Control Human gDNA at an input amount comparable to the samples.
   - **Negative control reactions/no template controls (NTCs):** assemble NTCs with 30 µl of Nuclease-Free Water or TE buffer (e.g., 10 mM Tris, 0.1 mM EDTA, pH 8.0).

   NOTE: The maximum volume of DNA cannot exceed 30 µl. For samples of less than 30 µl, add low TE buffer or Nuclease-Free Water to bring the total volume to 30 µl.

2. Prepare **1X PDF1** by diluting **10X PDF1** (light blue cap) in a 1:10 ratio using Nuclease-Free Water for the desired number of reactions and fragment size (IV.A.1.3). Mix by vortexing. Store on ice.

3. Using the previously determined optimal concentration from the **Enzyme dilution guide**, prepare the **Fragmentation enzyme dilution** as described below for the desired number of reactions and fragment size. Mix thoroughly by pipette. Store on ice.

Fragmentation enzyme dilution

Reagent	Cap color	Volume/reaction
1X PDF1	-	X µl
PEF1	Green	2 µl

4. Prepare **Template preparation D master mix** as described below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice.

Template preparation D master mix		
Reagent	**Cap color**	**Volume/reaction**
PBF1	Yellow	4 µl
PED1	Blue	1 µl
Fragmentation enzyme dilution	-	1 µl

NOTE: Prepare 10% excess to account for pipetting losses.

5. Assemble the **Template preparation reactions mixture** as shown in the table below. To each 30-µl sample from Step 1 above, add 6 µl of the **Template preparation D master mix**.

30 µl Sample or control
4 µl PBF1
1 µl PED1
1 µl Fragmentation enzyme dilution
36 µl Total volume

6. Mix thoroughly at least 10 times with a pipette set to 25 µl. Avoid introduction of air bubbles.
7. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
8. Centrifuge briefly to collect reaction contents at the bottom of each well.
9. Place the plate or tube(s) in a thermal cycler with a heated lid set to 101°C–105°C. Perform the **Template preparation reaction** using the conditions in the table below:

Template preparation reaction	
Temperature	**Time**
22°C	25 min
70°C	20 min
4°C	Hold for ≤2 hr

Keep sample tube on ice until the temperature of the thermal cycler reaches 22°C

10. After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly before placing on ice.
11. Proceed to **Library synthesis.**

NOTE: Following **Template preparation**, spin down reaction and continue to **Library synthesis** in the same plate or tube(s).

2. Library Synthesis

1. Prepare **Library synthesis D master mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Library synthesis D master mix		
Reagent	**Cap color**	**Volume/reaction**
SBD1	White	1 µl
SED1	White	4 µl

NOTE: Prepare 10% excess to allow for pipetting losses.

2. Remove the seal on the plate or open the tube(s).
3. To each well or tube, add 5 µl of the **Library synthesis D master mix**.

36 µl Template preparation reaction product
1 µl SBD1
4 µl SED1
41 µl Total volume

4. Mix thoroughly at least 10 times with a pipette set to 25 µl. Avoid introduction of air bubbles.
5. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
6. Centrifuge briefly to collect the contents at the bottom of each well or tube.
7. Return the plate or tube(s) to the thermal cycler with heated lid set to 101°C–105°C. Perform the **Library synthesis reaction** using the conditions in the table below:

Library synthesis reaction	
Temperature	**Time**
30°C	40 min
4°C	Hold for ≤30 min

8. After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly before placing on ice.
9. Proceed to **Library amplification.**

NOTE: Following the **Library synthesis** step, spin down reaction and continue to **Library amplification reaction** in the same plate or tube(s) maintained at 4°C.

3. Library Amplification

Multiple stages occur during **Library amplification** (see table in Step 8 below). During Stage 1 and Stage 2, stem-loop adapters are extended and cleaved. **Proper programming of the thermal cycler is critical for these first two steps to be completed correctly, with no denaturation step occurring until Stage 3.** Illumina-compatible indexes are incorporated into the template library in Stage 4 and the resulting template is amplified; the number of cycles required at this stage is dependent on the amount of input DNA used. In Stage 5, a final extension of the libraries occurs. Samples are cooled to 4°C in Stage 6, after which they are pooled and purified or stored at –20°C.

Selection of the optimal number of cycles for library amplification (Stage 4):
The number of PCR cycles required at Stage 4 of the **Library amplification** reaction is dependent on the amount of input DNA, fragmentation size, and thermal cycler used. Use the table below as a guide for selecting the number of PCR cycles.

Stage 4 amplification guide		
Input DNA	Number of cycles required to generate a 500–1,000-ng library	
	300-bp insert size	450-bp insert size
200 ng	5–6	6–7
100 ng	6–7	7–8
50 ng	7–8	8–9
5 ng	11–12	12–13

- **Optimization experiment:** An optimization experiment to identify the required number of PCR cycles is recommended. Use the desired amount of input DNA and allow the library amplification reaction to reach plateau. Determine the optimal number of amplification cycles and use this in the actual experiment.
- **Yield:** The amount of amplified library can range from 100 ng to 1 µg depending on many variables including sample type, fragment size, and thermal cycler used. When starting with Control Human gDNA with an average size of 300 bp or 450 bp over the recommended number of amplification cycles, typical yields range from 500 ng to 1 µg.

NOTE: Over-amplification could result in a higher rate of PCR duplicates in the library.

NOTE: It is critical to handle the index plate following the provided instructions to avoid cross-contamination of indexes. If the entire index plate will not be used, please refer to Appendix A for index plate handling instructions. No more than four freeze-thaw cycles are recommended for the index plate.

1. Prepare the Indexing Reagents described below:
   - Bring Indexing Reagents from freezer storage and thaw for 10 min on the bench top.
   - Spin index plate in a benchtop centrifuge to collect contents at the bottom of the wells.

2. Prepare **Library amplification D master mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Library amplification D master mix		
Reagent	**Cap color**	**Volume/reaction**
ABD1	Amber tube	42 µl
AED1	Violet	2 µl
Nuclease Free Water	Clear	5 µl

NOTES:
- Confirm ABD1 is fully thawed and thoroughly homogenized by heating briefly at 25°C and vortexing vigorously for 30 sec.
- Prepare 10% excess to allow for pipetting losses.

3. Remove the seal on the PCR plate or open the tube(s).
4. Add 49 µl of the **Library amplification D master mix** to each well or tube.
5. Add 10 µl of the appropriate Indexing Reagent to each well or tube.

Index plate precautions:

- Make sure the two corner notches of the index plate are on the left, and the barcode label on the long side of the index plate is facing you.
- Thoroughly wipe the index plate seal with 70% ethanol and allow it to dry to prevent cross-contamination.

41 µl Library synthesis reaction product
42 µl ABD1
2 µl AED1
5 µl Nuclease-Free Water
10 µl ThruPLEX HV UDI
100 µl Total volume

6. Mix thoroughly a minimum of 10 times with a pipette set to 70 µl. Avoid introduction of excessive air bubbles.
7. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s) and centrifuge briefly to collect the contents at the bottom of each well or tube.
8. Return the plate or tube(s) to the thermal cycler with heated lid set to 101°C–105°C. Perform the **Library amplification reaction** using the conditions in the table below.

CAUTION: Ensure that the thermal cycler does not have a denaturing step programmed until Stage 3.

Library amplification reaction				
	Stage	**Temperature**	**Time**	**# cycles**
Extension & cleavage	1 2	72°C 85°C	3 min 2 min	1 1
Denaturation	3	98°C	2 min	1
Library amplification	4	98°C 68°C	20 sec 75 sec	5–13 (see **Stage 4 amplification guide**)
Final extension	5	68°C	5 min	1
Hold	6	4°C	Hold	1

Stage 4 amplification guide		
Input DNA	Number of cycles required to generate a 500–1,000-ng library	
	300-bp insert size	450-bp insert size
200 ng	5–6	6–7
100 ng	6–7	7–8
50 ng	7–8	8–9
5 ng	11–12	12–13

9. Remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

NOTE: At this stage, samples can be immediately processed for next-generation sequencing (NGS) or stored frozen at –20°C for later processing. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to section IV.B.

B. Library Processing for Illumina Next-Generation Sequencing

1. Overview

This section contains guidelines for processing ThruPLEX DNA-Seq HV PLUS libraries for Illumina NGS. In some cases, recommended protocols are listed (**Library purification by AMPure XP beads or NucleoMag NGS Clean-up and Size Select**) while in others, general guidelines are given. For more information, contact technical support at technical_support@takarabio.com.

Libraries prepared from each sample will contain the specific indexes selected at the time of amplification. Once purified, the library should be quantified accurately prior to NGS to ensure efficient clustering on the Illumina flow cell. Instructions and recommendations on library purification, quantification, and quality are described in the following sections.

2. Library Purification by AMPure XP Beads or NucleoMag NGS Clean-up and Size Select Suspension

AMPure XP or NucleoMag NGS Clean-up and Size Select Suspension is the recommended method of library purification. Do not use QIAquick cleanup or other silica-based filters for purification as this will result in an incomplete removal of primers.

The ratio of beads to library DNA will determine the size-selection characteristics of the library. The ratio is also application dependent. For most NGS-based applications, a 1:1 bead-to-sample ratio is recommended. For more information, please refer to the vendor's recommendations on AMPure XP or NucleoMag NGS Clean-up and Size Select protocols for DNA purification.

Library purification reagents (supplied by the user)

Reagent
AMPure XP beads or NucleoMag NGS Clean-up and Size Select
Magnetic rack for 200-µl strip tubes
Freshly prepared 80% (v/v) ethanol
TE buffer, pH 8.0

AMPure XP and NucleoMag NGS Clean-up and Size Select protocol

NOTES:
- It is important to bring all the samples and reagents to room temperature.
- Always use freshly prepared 80% (v/v) ethanol for Step 3 and Step 4 below.
- Resuspend the AMPure XP reagent or NucleoMag NGS Clean-up and Size Select Suspension by gentle vortexing until no visible pellet is present at the bottom of the container.

1. In a 200-µl tube, mix 100 µl of AMPure XP reagent or NucleoMag NGS Clean-up and Size Select Suspension with 100 µl of amplified library, ensuring a 1:1 (v/v) ratio. Mix by pipette 10 times to achieve a homogeneous solution; incubate for 5 min at room temperature.
2. Pulse-spin the sample(s) on a bench top centrifuge and place the tube in a magnetic stand. Wait for at least 2 min or until the beads are completely bound to the side of the tube(s) and the solution is clear.
3. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard the supernatant. Add 200 µl of 80% (v/v) ethanol to the pellet and let stand for 30 sec.
4. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard the supernatant.
5. Repeat Steps 3 and 4 for a total of two ethanol washes.
6. Allow beads to air dry for no more than ~5 min—do not allow them to crack.
7. Elute the DNA by re-suspending the beads with 50 µl of 1 x TE buffer, pH 8.0. Pulse-spin the sample(s) using a low speed, bench top centrifuge and place it into a magnetic stand and let the beads bind to the side of the tube(s) completely (for 2 min) until the solution is clear.
8. While keeping the sample(s) in the magnetic stand and without disturbing the pellet, transfer the supernatant with a pipette into a new tube. If not used immediately, the purified library can be stored at –20°C.

3. Library Quantification and Quality Assessment

There are several approaches available for library quantification including real-time PCR with a library quantification kit for Illumina NGS libraries, such as the Library Quantification Kit (Takara Bio, Cat. No. 638324); fluorescence detection-based methods such as Qubit Fluorometer (Thermo Fisher Scientific) or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies); or a fragment analyzer, such as the Agilent Bioanalyzer.

A fragment analyzer, such as the Agilent Bioanalyzer, can also be used to assess the quality of the libraries. We recommend diluting an aliquot of each library in TE buffer to ~5 ng/µl. Load 1 µl of this diluted sample onto a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, Cat. No. 5067-4626). Libraries prepared using the ThruPLEX DNA-Seq HV PLUS result in a size distribution of library fragments that is dependent on the fragmentation protocol (Figure 4).

NOTE: Adapters added during ThruPLEX DNA-Seq HV PLUS library preparation result in an increase of approximately 140 bp in the size of each library.

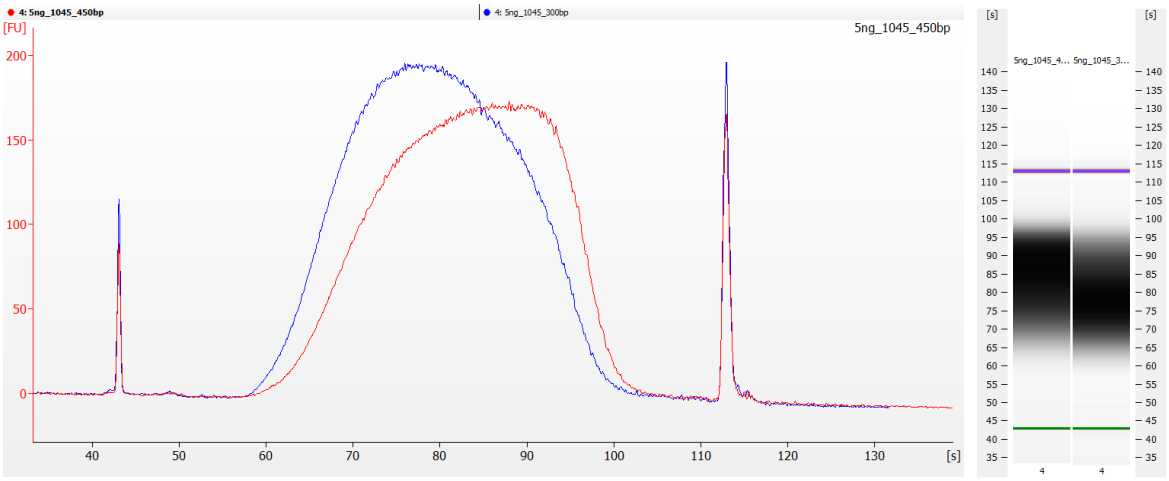

Figure 4. Bioanalyzer analysis of libraries prepared using ThruPLEX DNA-Seq HV PLUS. Libraries were prepared from 5 ng of Control Human gDNA using ThruPLEX DNA-Seq HV PLUS. Post library amplification, libraries were purified following the AMPure XP protocol (section IV.B.2). An aliquot of purified library was diluted to 5 ng/µl in TE buffer, and 1 µl of this diluted sample was loaded onto a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies). The blue trace is the library generated from the 300-bp protocol, and the red trace is the library generated from the 450-bp protocol.

Appendix A. Indexing Reagents

A. Overview

ThruPLEX DNA-Seq HV PLUS is paired with ThruPLEX HV UDI kits containing unique dual-indexed PCR primers for amplification of indexed Illumina-compatible NGS libraries. These kits contain indexed PCR primers offering up to 96 unique dual indexes for multiplexing samples. The indexed PCR primers are supplied pre-dispensed in 96-well plates and are available in two formats: a set of 96 unique dual indexes (Cat. No. R400738) and a set of 24 unique dual indexes (Cat. No. R400739) that represents a subset of Cat. No. R400738. Each well of the dual index plate is for single use. All indexes have been functionally validated to work with Illumina sequencing systems using two- or four-channel chemistry for base calling. They have not been validated with systems using one-channel chemistry. The table below summarizes the characteristics of the included Indexing Reagents, which consist of amplification primers containing Illumina-compatible indexes. Indexing Reagents should be stored at –20°C and should not be subjected to more than four freeze-thaw cycles.

NOTE: Indexing Reagents provided with ThruPLEX DNA-Seq HV PLUS cannot be substituted with indexing reagents from any other source.

B. Components

Store all components at –20°C.

Product name	Cat. No.	Concentration	Volume/tube
ThruPLEX HV UDI 1-24*	R400739	12.5 µM	12 µl
ThruPLEX HV UDI Set A	R400738	12.5 µM	12 µl

*The indexes in the ThruPLEX HV UDI 1-24 kit are a subset of the ThruPLEX HV UDI Set A kit.

C. ThruPLEX HV Unique Dual Index Sequences

The ThruPLEX HV unique dual indexes are 8-nt long and employ the “IDT for Illumina TruSeq® UD” i5 and i7 dual index sequences. An .xlsx file containing a full list of these indexes can be downloaded from our website.

Table 5. ThruPLEX HV UDI - Set A plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	U001	U009	U017	U025	U033	U041	U049	U057	U065	U073	U081	U089
B	U002	U010	U018	U026	U034	U042	U050	U058	U066	U074	U082	U090
C	U003	U011	U019	U027	U035	U043	U051	U059	U067	U075	U083	U091
D	U004	U012	U020	U028	U036	U044	U052	U060	U068	U076	U084	U092
E	U005	U013	U021	U029	U037	U045	U053	U061	U069	U077	U085	U093
F	U006	U014	U022	U030	U038	U046	U054	U062	U070	U078	U086	U094
G	U007	U015	U023	U031	U039	U047	U055	U063	U071	U079	U087	U095
H	U008	U016	U024	U032	U040	U048	U056	U064	U072	U080	U088	U096

Table 6. ThruPLEX HV UDI sequences.

Index	i7 bases for sample sheet	i5 bases for sample sheet (MiSeq®, NovaSeq™, HiSeq® 2000/2500)	i5 bases for sample sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U001	CCGCGGTT	AGCGCTAG	CTAGCGCT
U002	TTATAACC	GATATCGA	TCGATATC
U003	GGACTTGG	CGCAGACG	CGTCTGCG
U004	AAGTCCAA	TATGAGTA	TACTCATA
U005	ATCCACTG	AGGTGCGT	ACGCACCT
U006	GCTTGTCA	GAACATAC	GTATGTTC
U007	CAAGCTAG	ACATAGCG	CGCTATGT
U008	TGGATCGA	GTGCGATA	TATCGCAC
U009	AGTTCAGG	CCAACAGA	TCTGTTGG
U010	GACCTGAA	TTGGTGAG	CTCACCAA
U011	TCTCTACT	CGCGGTTC	GAACCGCG
U012	CTCTCGTC	TATAACCT	AGGTTATA
U013	CCAAGTCT	AAGGATGA	TCATCCTT
U014	TTGGACTC	GGAAGCAG	CTGCTTCC
U015	GGCTTAAG	TCGTGACC	GGTCACGA
U016	AATCCGGA	CTACAGTT	AACTGTAG
U017	TAATACAG	ATATTCAC	GTGAATAT
U018	CGGCGTGA	GCGCCTGT	ACAGGCGC
U019	ATGTAAGT	ACTCTATG	CATAGAGT
U020	GCACGGAC	GTCTCGCA	TGCGAGAC
U021	GGTACCTT	AAGACGTC	GACGTCTT
U022	AACGTTCC	GGAGTACT	AGTACTCC
U023	GCAGAATT	ACCGGCCA	TGGCCGGT
U024	ATGAGGCC	GTTAATTG	CAATTAAC
U025	ACTAAGAT	AACCGCGG	CCGCGGTT
U026	GTCGGAGC	GGTTATAA	TTATAACC
U027	CTTGGTAT	CCAAGTCC	GGACTTGG
U028	TCCAACGC	TTGGACTT	AAGTCCAA
U029	CCGTGAAG	CAGTGGAT	ATCCACTG
U030	TTACAGGA	TGACAAGC	GCTTGTCA
U031	GGCATTCT	CTAGCTTG	CAAGCTAG
U032	AATGCCTC	TCGATCCA	TGGATCGA
U033	TACCGAGG	CCTGAACT	AGTTCAGG
U034	CGTTAGAA	TTCAGGTC	GACCTGAA
U035	AGCCTCAT	AGTAGAGA	TCTCTACT
U036	GATTCTGC	GACGAGAG	CTCTCGTC
U037	TCGTAGTG	AGACTTGG	CCAAGTCT
U038	CTACGACA	GAGTCCAA	TTGGACTC
U039	TAAGTGGT	CTTAAGCC	GGCTTAAG
U040	CGGACAAC	TCCGGATT	AATCCGGA
U041	ATATGGAT	CTGTATTA	TAATACAG

Index	i7 bases for sample sheet	i5 bases for sample sheet (MiSeq, NovaSeq, HiSeq 2000/2500)	i5 bases for sample sheet (MiniSeq, NextSeq, HiSeq 3000/4000)
U042	GCGCAAGC	TCACGCCG	CGGCGTGA
U043	AAGATACT	ACTTACAT	ATGTAAGT
U044	GGAGCGTC	GTCCGTGC	GCACGGAC
U045	ATGGCATG	AAGGTACC	GGTACCTT
U046	GCAATGCA	GGAACGTT	AACGTTCC
U047	GTTCCAAT	AATTCTGC	GCAGAATT
U048	ACCTTGGC	GGCCTCAT	ATGAGGCC
U049	ATATCTCG	ATCTTAGT	ACTAAGAT
U050	GCGCTCTA	GCTCCGAC	GTCGGAGC
U051	AACAGGTT	ATACCAAG	CTTGGTAT
U052	GGTGAACC	GCGTTGGA	TCCAACGC
U053	CAACAATG	CTTCACGG	CCGTGAAG
U054	TGGTGGCA	TCCTGTAA	TTACAGGA
U055	AGGCAGAG	AGAATGCC	GGCATTCT
U056	GAATGAGA	GAGGCATT	AATGCCTC
U057	TGCGGCGT	CCTCGGTA	TACCGAGG
U058	CATAATAC	TTCTAACG	CGTTAGAA
U059	GATCTATC	ATGAGGCT	AGCCTCAT
U060	AGCTCGCT	GCAGAATC	GATTCTGC
U061	CGGAACTG	CACTACGA	TCGTAGTG
U062	TAAGGTCA	TGTCGTAG	CTACGACA
U063	TTGCCTAG	ACCACTTA	TAAGTGGT
U064	CCATTCGA	GTTGTCCG	CGGACAAC
U065	ACACTAAG	ATCCATAT	ATATGGAT
U066	GTGTCGGA	GCTTGCGC	GCGCAAGC
U067	TTCCTGTT	AGTATCTT	AAGATACT
U068	CCTTCACC	GACGCTCC	GGAGCGTC
U069	GCCACAGG	CATGCCAT	ATGGCATG
U070	ATTGTGAA	TGCATTGC	GCAATGCA
U071	ACTCGTGT	ATTGGAAC	GTTCCAAT
U072	GTCTACAC	GCCAAGGT	ACCTTGGC
U073	CAATTAAC	CGAGATAT	ATATCTCG
U074	TGGCCGGT	TAGAGCGC	GCGCTCTA
U075	AGTACTCC	AACCTGTT	AACAGGTT
U076	GACGTCTT	GGTTCACC	GGTGAACC
U077	TGCGAGAC	CATTGTTG	CAACAATG
U078	CATAGAGT	TGCCACCA	TGGTGGCA
U079	ACAGGCGC	CTCTGCCT	AGGCAGAG
U080	GTGAATAT	TCTCATTC	GAATGAGA
U081	AACTGTAG	ACGCCGCA	TGCGGCGT
U082	GGTCACGA	GTATTATG	CATAATAC
U083	CTGCTTCC	GATAGATC	GATCTATC

Index	i7 bases for sample sheet	i5 bases for sample sheet (MiSeq, NovaSeq, HiSeq 2000/2500)	i5 bases for sample sheet (MiniSeq, NextSeq, HiSeq 3000/4000)
U084	TCATCCTT	AGCGAGCT	AGCTCGCT
U085	AGGTTATA	CAGTTCCG	CGGAACTG
U086	GAACCGCG	TGACCTTA	TAAGGTCA
U087	CTCACCAA	CTAGGCAA	TTGCCTAG
U088	TCTGTTGG	TCGAATGG	CCATTCGA
U089	TATCGCAC	CTTAGTGT	ACACTAAG
U090	CGCTATGT	TCCGACAC	GTGTCGGA
U091	GTATGTTC	AACAGGAA	TTCCTGTT
U092	ACGCACCT	GGTGAAGG	CCTTCACC
U093	TACTCATA	CCTGTGGC	GCCACAGG
U094	CGTCTGCG	TTCACAAT	ATTGTGAA
U095	TCGATATC	ACACGAGT	ACTCGTGT
U096	CTAGCGCT	GTGTAGAC	GTCTACAC

Appendix B. Troubleshooting Guide

Table 7. Troubleshooting guide for ThruPLEX DNA-Seq HV PLUS.

Problem	Potential cause	Suggested solutions
Sample amplification appears similar to no template control (NTC) amplification, or does not produce amplified product	No input DNA added	Quantify input before using the kit
	Incorrect library template used (e.g., RNA, ssDNA)	Adhere to DNA sample requirements (section III.A)
NTC produces a yield similar to sample reaction products	NTC contaminated with DNA	Use a fresh control sample and check all reagents; replace kit if necessary. Clean area thoroughly and use PCR-dedicated plastics and pipettes.
After purification of the amplified library, Bioanalyzer traces show multiple peaks besides the markers	Input sample contains unevenly fragmented DNA of various sizes	If possible, quantify and check input DNA prior to using the kit. Sequencing is still recommended.
After purification of the amplified library, Bioanalyzer traces show broad peak(s) extending from 1,000 bp to greater than 10,000 bp.	Library was overamplified or the Bioanalyzer chip was overloaded. (This is common with high-sensitivity chips.)	Perform fewer PCR cycles during the Library Amplification Reaction. For high-sensitivity chips, load ~1–5 ng/µl. Repeat the Bioanalyzer run.
	Incorrect dilution of PEF1	Double-check PEF1 concentration used. Optimize dilution concentration with the Control Human gDNA and/or your control dsDNA sample.

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Customer Service/Ordering	**Technical Support**
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fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
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