Takara Bio USA

ThruPLEX® Tag-Seq HV User Manual

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(022720)

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I. Introduction

A. Overview

The ThruPLEX Tag-Seq HV kit is designed to provide up to 96 indexed libraries for higher multiplexing capabilities on Illumina® NGS platforms. ThruPLEX Tag-Seq HV 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 of fragmented double-stranded DNA 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 and no sample transfers are necessary, thus preventing handling errors and loss of valuable samples. With high library diversity, ThruPLEX Tag-Seq HV libraries excel when combined with target enrichment and deliver high-quality sequencing results.

Pairing ThruPLEX Tag-Seq HV 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/or other enrichment techniques. It is ideally suited for use with small fragments of DNA such as cell-free plasma DNA or damaged DNA from formalin-fixed paraffin-embedded (FFPE) tissue.

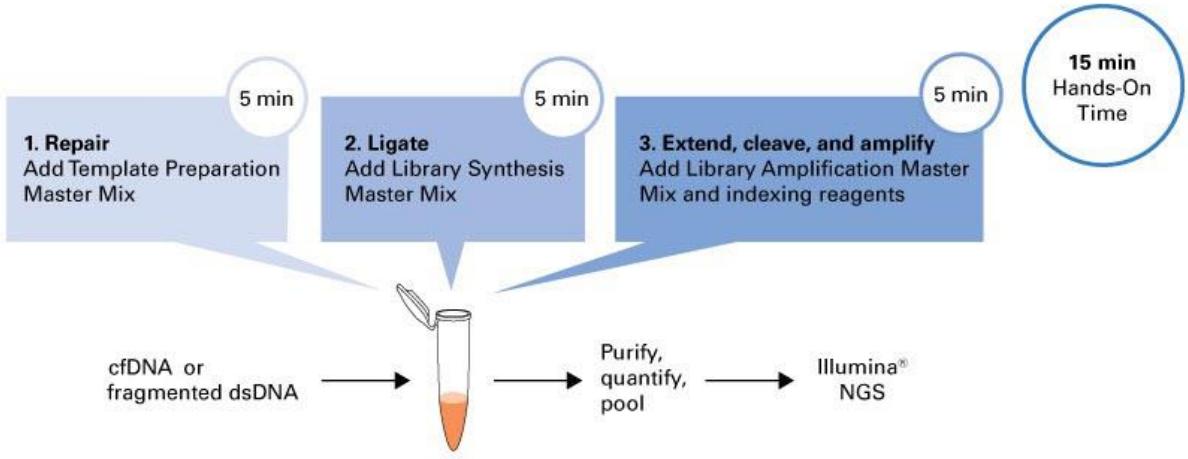

Figure 1. ThruPLEX Tag-Seq HV single-tube library preparation workflow. The ThruPLEX Tag-Seq HV workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify or transfer the sample material.

B. Principle

The ThruPLEX Tag-Seq HV kit is based on our patented ThruPLEX HV 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 repaired and yields molecules with 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-strand tails, both of which contribute to nonspecific background found with many other NGS preparations. In the final step, the 3' ends of the 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.

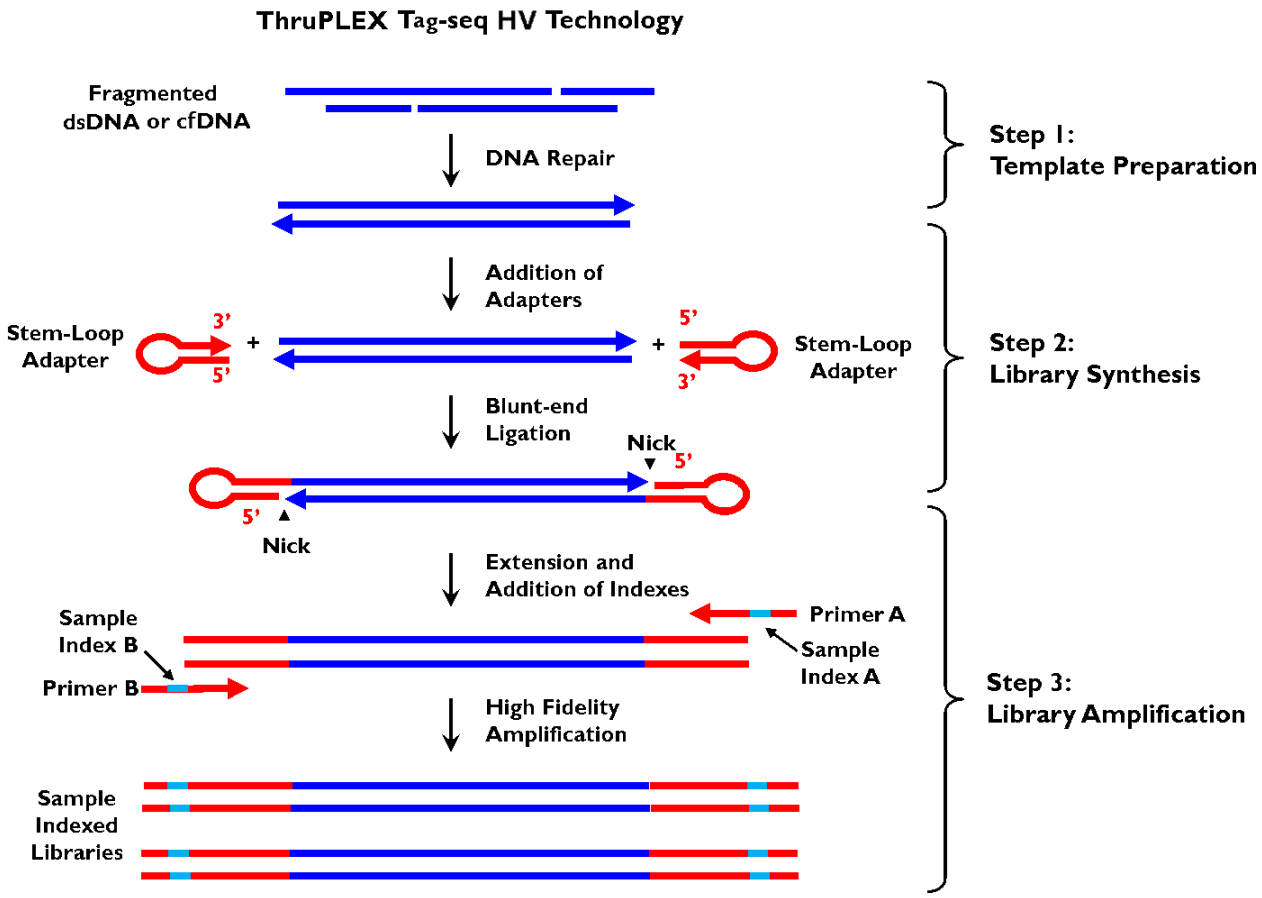

Figure 2. ThruPLEX Tag-Seq HV technology uses a three-step, single-tube reaction that starts with fragmented double-stranded DNA or cfDNA (5 ng to 200 ng). Stem-loop adapters with molecular tags (not shown) are blunt-end ligated to repaired input DNA. These molecules are extended, then amplified to include barcodes using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX Tag-Seq HV molecular tags

Research in Next Generation Sequencing (NGS) is rapidly evolving, and the ability to confidently detect low-frequency alleles or discriminate between molecules is now critical to the development of highly sensitive, NGS-based assays frequently used in cancer research, developmental research involving the fetal fraction derived from maternal plasma and other fields. Library preparation is a critical step in the NGS workflow and has a direct impact on the quality of sequencing results. This process involves placing Illumina-compatible sequencing adapters with unique molecular identifiers (UMI) on DNA fragments and adding Illumina-compatible unique dual indexes (UDI) for identification of individual samples. Multiple samples are then pooled and sequenced in parallel.

There are several key challenges when detecting low-frequency alleles and differentiating DNA fragments through sequencing. First, different sample types, such as cfDNA, have limited, highly-degraded material that can make generating high-quality libraries difficult. A protocol in which the sample never leaves the tube is advantageous to ensure accurate sample tracking and to avoid contamination, which would lead to false positives. The three-step, single-tube library preparation workflow (Figure 1) is the simplest in the industry, minimizing handling errors, loss of valuable samples and is automation friendly.

Furthermore, PCR artifacts can be introduced during library preparation and errors can occur during sequencing, both of which are known as false positives. The commitment to overcome these challenges is the core of the ThruPLEX Tag-Seq HV kit. The ThruPLEX adapters have been redesigned to include discrete unique molecular identifiers (UMI). Each kit contains 144 unique sequences used to "tag" DNA molecules and track the fragments through the library preparation. This allows detection of low-frequency alleles and the ability to differentiate between molecules at high sensitivity and specificity. The performance of the libraries is highly reproducible between replicates, sequencing runs and from sample to sample.

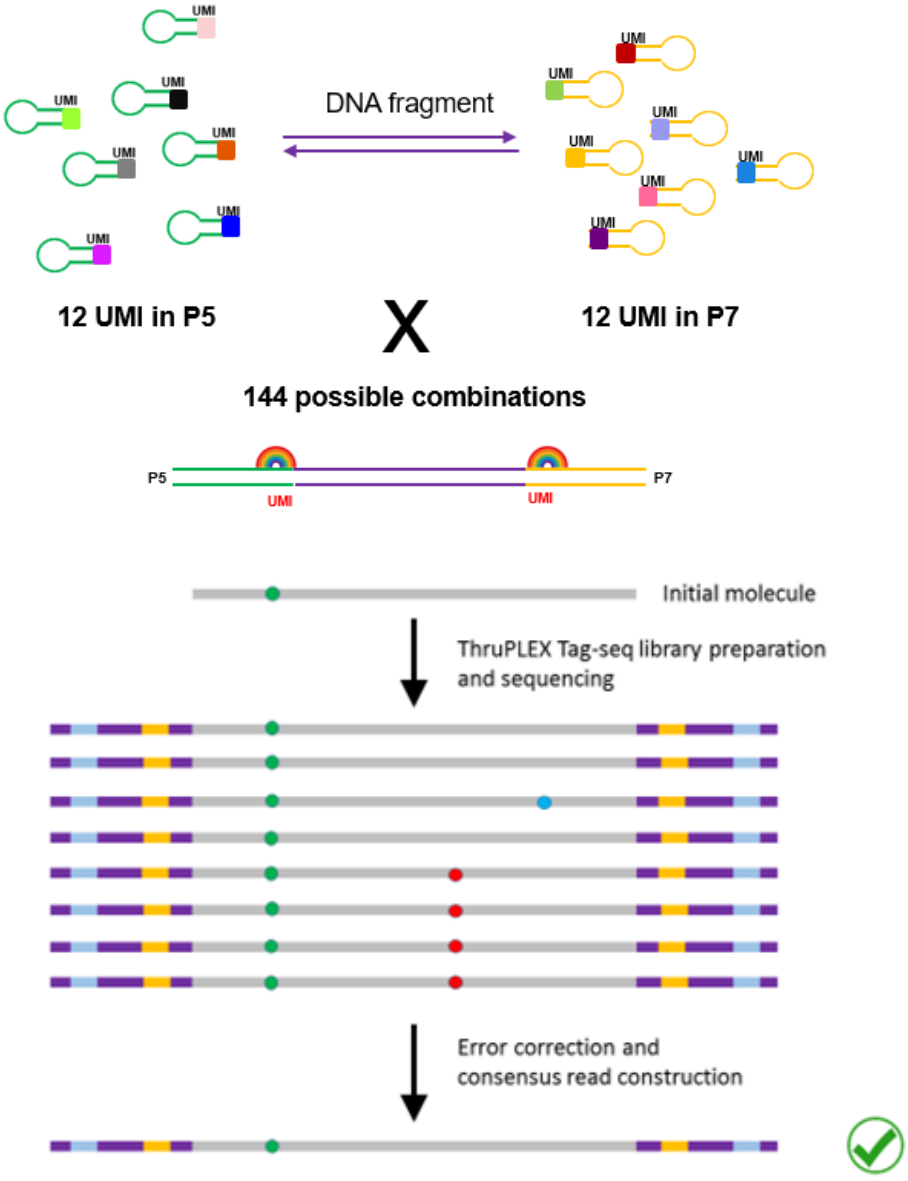

Figure 3. ThruPLEX Tag-Seq HV contains 144 discrete unique molecular identifiers (UMIs). The ThruPLEX Tag-Seq HV is designed to remove the ambiguity in variant calling by reducing the false positive calls coming from DNA polymerase and sequencing errors. The seven base UMIs are located at the beginning of the reads ensuring an easy demultiplexing of the samples to simplify the analysis

D. ThruPLEX Tag-Seq HV Workflow

The ThruPLEX Tag-Seq HV workflow is highly streamlined (Figure 4) and consists of the following three steps:

- **Template Preparation** for efficient repair of the fragmented double-stranded DNA input.
- **Library Synthesis** for ligation of our patented stem-loop adapters.
- **Library Amplification** for extension of the template 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 Tag-Seq HV workflow takes place in a single tube or well and is completed in about two hours.

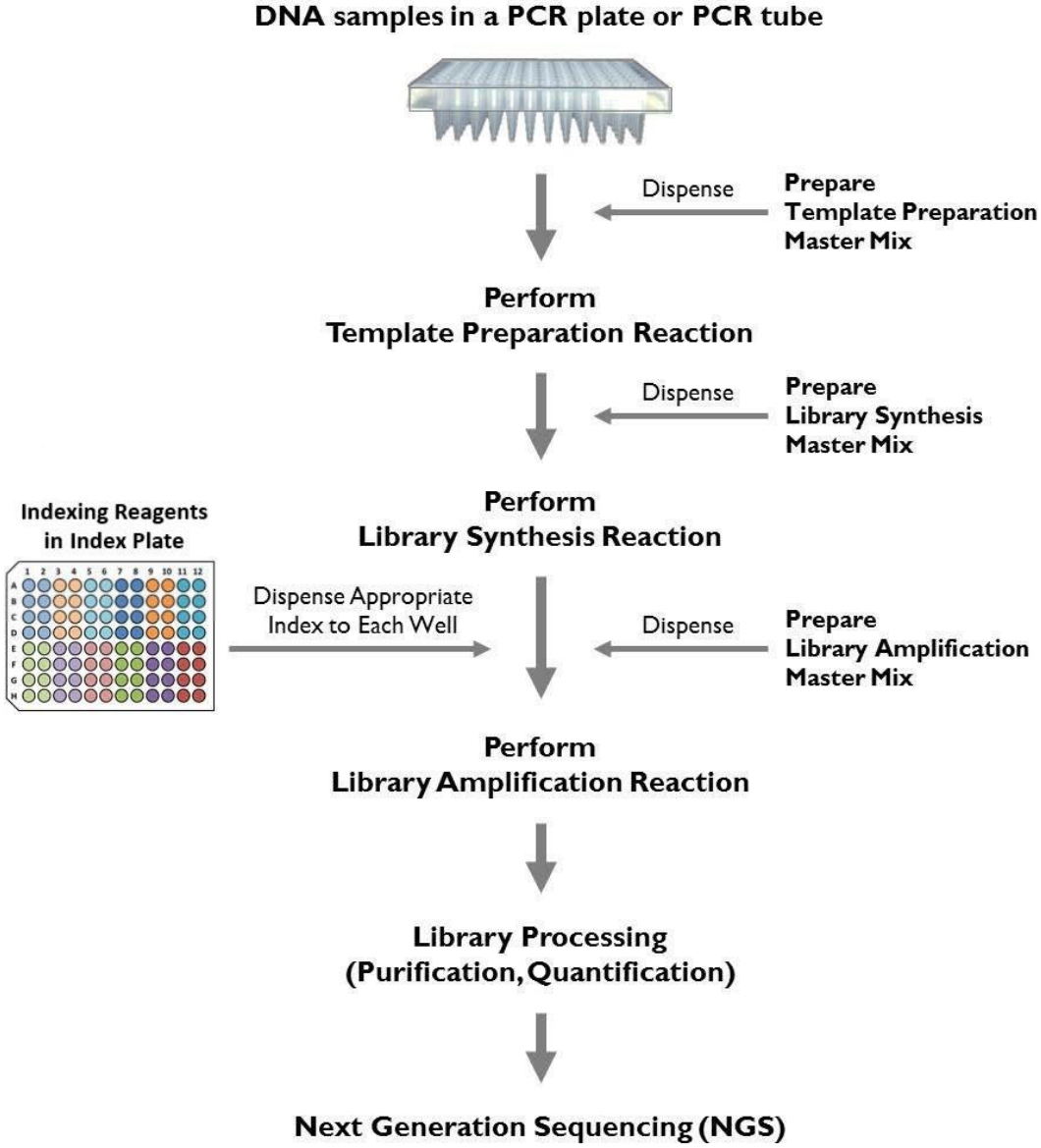

Figure 4. Overview of ThruPLEX Tag-Seq HV library preparation for Illumina NGS, starting with fragmented DNA.

II. List of Components

The ThruPLEX Tag-Seq HV kit contains sufficient reagents to prepare up to 24 or 96 reactions. Enough buffers and enzymes are provided to prepare master mixes with additional 10% volume. **Freeze-thaw of reagents should be limited to no more than six times**. Contents of the ThruPLEX Tag-Seq HV kit are not interchangeable with other Takara Bio products.

A. Components

Table I. ThruPLEX Tag-Seq HV kit contents.

Name	Cap color	Storage	R400742 (24 rxns)	R400743 (96 rxns)
Control Fragmented Human gDNA (5 ng/µl)	N/A	–20°C	1 tube	1 tube
PBT1	Blue	–20°C	1 tube	1 tube
PET1	Blue	–20°C	1 tube	1 tube
SBT1	White	–20°C	1 tube	1 tube
SET1	White	–20°C	1 tube	1 tube
ABT1	Violet	–20°C	1 tube	4 tubes
AET1	Violet	–20°C	1 tube	1 tube
Nuclease-Free Water	Clear	–20°C	1 tube	1 tube
ThruPLEX HV UDI*		–20°C	1 Dual Index Plate (24 UDI)	1 Dual Index Plate (96 UDI)

*included in bundle part numbers R400742 & R400743; also sold separately as R400738 & R400739

B. Shipping and Storage Conditions

ThruPLEX Tag-Seq HV is shipped on dry ice. The kit should be stored at –20°C upon arrival.

C. Additional Materials Required

The following reagents and equipment are required but not supplied. These items have been validated to work with this protocol.

- ThruPLEX HV Indexing module (part number R400738 or R400739) if ThruPLEX Tag-Seq HV was purchased as a core components kit
- 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
- Single-channel pipette: 10 µl, 20 µl, and 200 µl, 1000 µl
- Multi-channel pipettes: 20 µl and 200 µl
- Low-binding filter pipette tips: 10 µl, 20 µl, 200 µl, 1000 µl
- Low-binding aerosol barrier tips 10 µl, 200 µl, 1000 µl
- TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)
- Magnetic rack
- Freshly prepared 80% (v/v) ethanol
- Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880)

NOTE: Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly recommend aliquoting the beads upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This aliquoting process 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)
- Bioanalyzer or TapeStation, for library size distribution

III. General Considerations

A. Sample Requirements

Table II. DNA sample requirements.

DNA sample requirements	
Source	Plasma, cells, urine, or other biofluids, gDNA, FFPE
Type	Isolated cell-free DNA or fragmented double-stranded DNA
Recommended 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 fragmented dsDNA in order to be used with ThruPLEX Tag-Seq HV. Fragmented double-stranded DNA (gDNA), degraded DNA from sources such as FFPE, cfDNA from plasma, or other biofluids are suitable. This kit is not for use with single-stranded DNA (ssDNA) or RNA.

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 III. Table 3II. Recommended DNA Purification Kits.

Sample type	Recommended kit	Catalog Nos.
FFPE tissue	NucleoSpin DNA FFPE XS	740980.10, 740980.50, 740980.250
Plasma, urine, etc.	NucleoSnap DNA Plasma	740300.10, 740300.50
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. When working with cfDNA, quantification of the mononucleosomal cfDNA fragments by Bioanalyzer run is recommended. Use an appropriate input amount of DNA to ensure sufficient variant copies are available for the library preparation process to achieve the desirable detection sensitivity. In general, detection of alleles present at low frequencies requires higher input amount of DNA.

Table IV. Estimated gene 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 plasma samples is highly variable. All numbers are rounded down to the nearest whole number.

4. Input Volume

The maximum input sample volume is 30 µl. If a sample is in a larger volume, the DNA must be concentrated into 30 µl or less. Care should be taken to ensure the buffer concentration is appropriate (see below).

5. Input Buffer

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

6. Fragment Size

The optimal DNA fragment size is between 150 and 500 bp. The ThruPLEX Tag-Seq HV Kit is a ligation-based technology and adapters added during the process result in an approximately 140-bp increase in the size of each DNA template fragment. Library molecules with shorter inserts (200–300 bp) tend to cluster and amplify more efficiently on the Illumina flow cell. Depending on the application and requirements, the AMPure purification step following the final step (Library Amplification) can be replaced with a size-selection step to remove unwanted fragments.

7. Using Cell-Free DNA from Plasma

Cell-free DNA (cfDNA) isolated from plasma samples contains both high and low molecular weight DNA fragments (Figure 5). The composition and concentration of the isolated cfDNA differ from sample to sample and may vary depending on the isolation method used. The cfDNA species of most interest is the mononucleosomal DNA fragments of about 170 bp in length; the concentration can be determined using a fragment analyzer such as the Agilent Bioanalyzer.

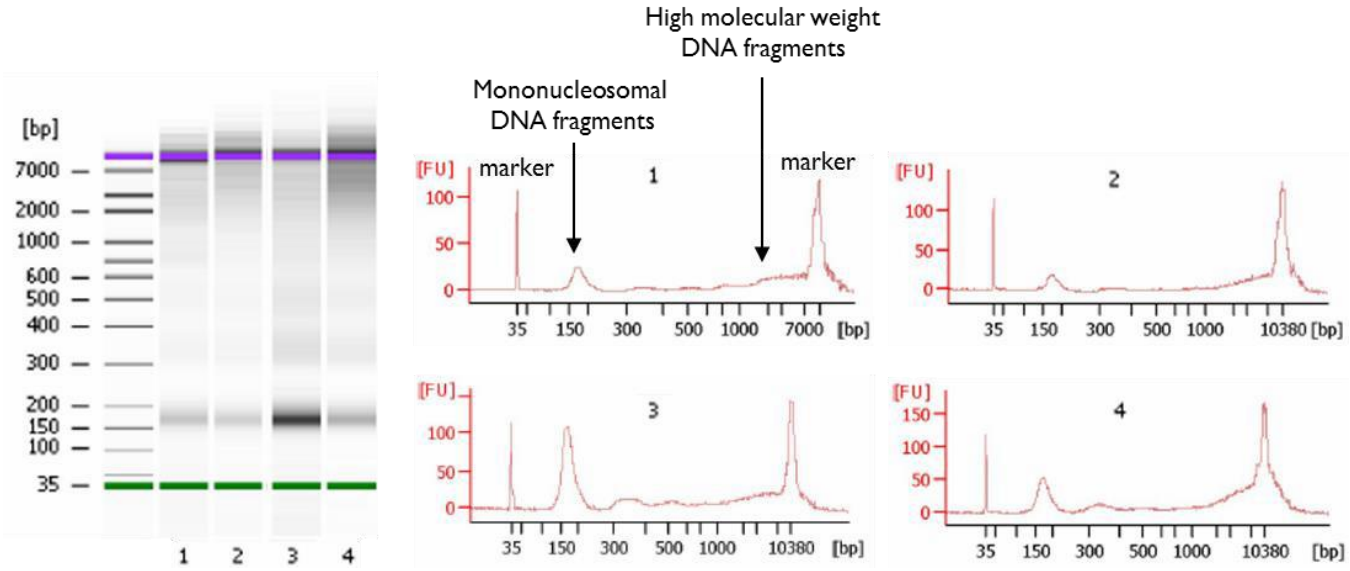

Figure 5. Fragment size distribution of cfDNA isolated from plasma. cfDNA from four different human plasma samples was isolated and analyzed using the Agilent Bioanalyzer. Distinct features of the isolated cfDNA is a peak (or band) centered around 170 bp and higher molecular weight fragments.

8. 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 Fragmented Human gDNA, included in the kit). Include a negative control (No Template Control, NTC) with low-EDTA 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 handle 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 the 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, while 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 use of the ThruPLEX HV UDI kits for use with ThruPLEX Tag-Seq HV. The indexing modules are available to be purchased as a separate part or bundled with the core enzymatic components in a kit. They are available in 24-reaction or 96-reaction kit sizes and are available as unique dual indexes.

NOTE: The ThruPLEX DNA-Seq HV 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 at the ThruPLEX DNA-Seq HV Product Page, under the Resources tab. Before starting the ThruPLEX DNA-seq HV Library Preparation Protocol (Section IV.A), refer to Appendix A for information on index sequences, Index Plate handling instructions, and multiplexing and index-pooling guidelines.

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 pipet tip to collect the required amount of 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

Make sure the latest version of the Illumina Experiment Manager (IEM) is installed (version 1.18.1 or later). Prior to starting the ThruPLEX Tag-Seq HV 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

ThruPLEX Tag-Seq HV is compatible with major target enrichment products. ThruPLEX Tag-Seq HV target enrichment protocols can be accessed through the Learning Center at takarabio.com.

6. Sequencing Depth

In addition to input amount, sequencing depth is another factor that determines detection sensitivity. While sufficient variant molecules must be present in the input DNA to be detected downstream, sufficient coverage is required to utilize the unique molecular tags in ThruPLEX Tag-Seq HV libraries to build consensus sequences. In general, detection of alleles present at lower frequencies requires sequencing to a higher depth. The prerequisite to higher depth of sequencing is a high complexity library that allows new reads to be found (non-saturation). ThruPLEX Tag-Seq HV offers this quality in a consistent way for every sample.

Table V. Sequencing depth.

Estimated mean raw sequencing depth required*			
Minimum number of unique variant molecules to make a confident call	Allele frequency		
	5%	1%	0.5%
3	600X	3,000X	6,000X
5	1,000X	5,000X	10,000X
10	2,000X	10,000X	20,000X

*Raw sequencing depth includes all reads prior to removal of duplicates. This is calculated using a target peak amplification family size of 10 reads per unique molecule.

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 contamination during the sample preparation and subsequent amplification reactions. For more information please refer to the appropriate Material Safety Data Sheets (MSDS) available online at takarabio.com.

IV. Protocols

A. Protocol: ThruPLEX Tag-Seq HV Library Preparation

1. Template Preparation

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 used.

1. Prepare samples as described below:

   - **Starting material:** 5–200 ng fragmented DNA
   - **Samples:** Dispense 30 µl of cfDNA or fragmented doubled-stranded DNA (167 pg/µl–6.7 ng/µl) into each PCR tube or well of a PCR plate.
   - **Positive control reactions using reference DNA:** If necessary, assemble reactions using 30 µl of the included Control Fragmented Human gDNA at an input amount comparable to the samples.
   - **Negative control reactions/No Template Controls (NTCs):** If necessary, assemble NTCs with 30 µl of nuclease-free water or low-EDTA TE buffer (e.g., 10 mM Tris, 0.1 mM EDTA, pH 8.0).

NOTE: The maximum volume of DNA cannot exceed 30 µl.

2. Prepare **Template Preparation Master Mix** as described in the table below for the desired number of reactions, plus 10% excess. Mix thoroughly with a pipette. Keep on ice until used:

Template Preparation Master Mix		
Reagent	**Cap color**	**Volume/reaction**
PBT1	Blue	5 µl
PET1	Blue	1 µl

3. Assemble the **Template Preparation Reaction Mixture** as shown in the table below. To each 30-µl sample from Step 1 above, add 6 µl of the **Template Preparation Master Mix**.

Template Preparation Reaction Mixture	
Component	**Volume/reaction**
Sample or Control (fragmented DNA input)	30 µl
Template Preparation Master Mix	6 µl
Total volume	36 µl

4. Mix thoroughly with a pipette. 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 ensure the entire volume of the reaction is collected at the bottom of each well or tube.
7. Place the plate or tube(s) in a thermal cycler with 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
55°C	20 min
4°C	Hold for ≤2 hr

8. After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly.
9. Proceed to the **Library Synthesis** step in the same plate or tube(s) maintained at 4°C.

2. Library Synthesis

1. Prepare **Library Synthesis Master Mix** on ice as described in the table below for the desired number of reactions, plus 10% excess. Mix thoroughly with a pipette. Keep on ice until used.

Library Synthesis Master Mix		
Reagent	**Cap color**	**Volume/reaction**
SBT1	White	2 µl
SET1	White	4 µl

2. Remove the plate seal or open the tube(s) containing the Template Preparation Reaction Product.
3. Assemble the **Library Synthesis Reaction Mixture** as shown in the table below. To each well or tube, add 6 µl of the **Library Synthesis Master Mix**.

Library Synthesis Reaction Mixture	
Component	**Volume/reaction**
Template Preparation Reaction Product	36 µl
Library Synthesis Master Mix	6 µl
Total volume	42 µl

4. Mix thoroughly with a pipette. 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.
9. Proceed to the **Library Amplification** step in the same plate or tube(s) maintained at 4°C.

3. Library Amplification Step

1. Prepare the ThruPLEX HV UDI as described below:
   - Remove the index plate from the freezer and thaw for ten min on the bench top
   - Spin the plate in a tabletop centrifuge to collect contents at the bottom of the well
2. Prepare **Library Amplification Master Mix** as described in the table below for the desired number of reactions, plus 10% excess. Mix thoroughly with a pipette. Keep on ice until used.

Library Amplification Master Mix		
Reagent	**Cap color**	**Volume/reaction**
ABT1	Violet	46 µl
AET1	Violet	2 µl

3. Assemble the **Library Amplification Reaction Mixture** as shown in the table below. To each well or tube, add 48 µl of the **Library Amplification Master Mix** and 10 µl of a unique ThruPLEX HV UDI.

Library Amplification Reaction Mixture	
Component	**Volume/reaction**
Template Preparation Reaction Product	42 µl
Library Amplification Master Mix	48 µl
ThruPLEX HV UDI	10 µl
Total volume	100 µl

4. 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	1	72°C	3 min	1
	2	85°C	2 min	1
Denaturation	3	98°C	2 min	1
Library amplification	4	98°C 65°C	20 sec 75 sec	5–11 (see Stage 4 Amplification Guide)
Final extension		68°C	5 min	
Hold	5	4°C	Hold	1

Stage 4 Amplification Guide	
Input DNA	Number of cycles required to generate 500–1,000 ng library
200 ng	5–6
100 ng	6–7
50 ng	7–8
10 ng	9–10
5 ng	10–11

5. Remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.
6. Transfer the samples to a post-PCR work station for library purification.

B. Library Purification (post-PCR workstation)

Reagents (to be supplied by the user): AMPure XP beads, Magnetic rack for 1.5-ml centrifuge tubes, freshly prepared 80% (v/v) ethanol, TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)

NOTES:

- It is important to bring all samples and reagents to be used to room temperature
- Always use freshly prepared 80% (v/v) ethanol for Step 6 (below)
- Resuspend the AMPure XP reagent by gentle vortexing until no visible pellet is present at the bottom of the container.

1. Vortex room-temperature AMPure XP beads until evenly mixed, then add 100 µl of AMPure XP beads to each sample.
2. Mix thoroughly by vortexing for 30 seconds.
3. Incubate at room temperature for 5 min.
4. Briefly spin the samples to collect the liquid from the sides of the sample tubes or wells. Please the samples on the magnetic separation device for ~5 min or until the liquid appears completely clear.
5. While the samples are on the magnetic separation device, pipette the supernatant and discard.
6. Keep the samples on the magnetic separation device. Add 200 µl of freshly prepared 80% ethanol to each sample without disturbing the beads. Wait for 30 seconds and then carefully discard the supernatant.
7. Repeat the ethanol wash (Step 6) once.

8. Briefly spin the samples to collect the liquid from the sides of the sample tubes or wells. Place the samples on the magnetic separation device, then remove all remaining ethanol with a fine pipette tip.
9. Let the sample dry for approximately 2 min, or until the pellet is no longer shiny, but before a crack appears.
10. Once the beads are dry, remove the samples from the magnetic separation device and add 50 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) or nuclease-free water to cover the bead pellet. Mix thoroughly by vortexing to resuspend the beads. Incubate at room temperature for 2 min to rehydrate.
11. Briefly spin the samples to collect the liquid from the sides of the sample tubes or wells. Place the samples back on the magnetic separation device for 2 min, or until the solution is completely clear.
12. Transfer the clear supernatant containing purified libraries from each well to a nuclease-free, low-adhesion tube. Label each tube with sample information. The purified libraries can be stored at –20°C.

C. ThruPLEX Tag-Seq HV Library Quantification and Quality Assessment

Quantification of the libraries can be performed using fluorescence detection-based methods, Qubit Fluorometer (Thermo Fisher Scientific), or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). The 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 a 1-µl aliquot of this diluted sample onto a Bioanalyzer high-sensitivity DNA chip (Agilent Technologies, Cat. No. 5067-4626).

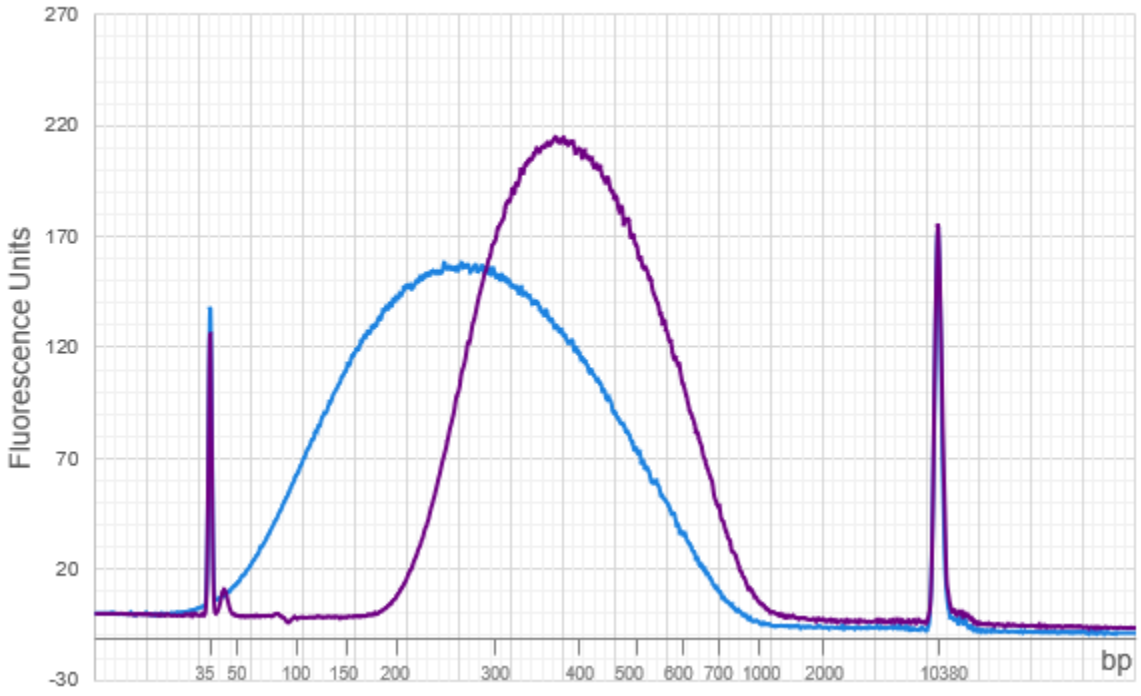

Figure 6. Bioanalyzer traces from 250-bp control Covaris-sheared genomic DNA before and after library preparation. The ThruPLEX Tag-Seq HV library (purple) was generated from ~250-bp sheared gDNA (blue).

Appendix A. Indexing Reagents

A. Overview

ThruPLEX Tag-Seq HV Kits are 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. Table 3 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 Tag-Seq HV kit 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 VI. 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 VII. 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 VIII. Troubleshooting Guide for the ThruPLEX Tag-Seq HV Kits

Problem	Potential Cause	Suggested Solutions
Sample amplification curve looks like No Template Control (NTC) amplification curve or does not produce amplified product	No input DNA added	Quantitate input before using the kit
	Incorrect library template used (e.g., RNA, ssDNA)	Adhere to DNA Sample Requirements (Section III.A)
NTC amplification curve appears early or 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 shows multiple peaks besides the markers	Input sample contains unevenly fragmented DNA of various sizes (e.g., plasma DNA)	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.

Contact Us	
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