Takara Bio USA

ThruPLEX® Tag-Seq FLEX User Manual

Cat. Nos. R400734 & R400735
(112024)

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I. Introduction

A. Overview

ThruPLEX Tag-Seq FLEX 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 1 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 FLEX libraries excel when combined with target enrichment and deliver high-quality sequencing results.

Pairing ThruPLEX Tag-Seq FLEX with unique dual indexes (UDIs) allows for multiplexing of up to 384 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.

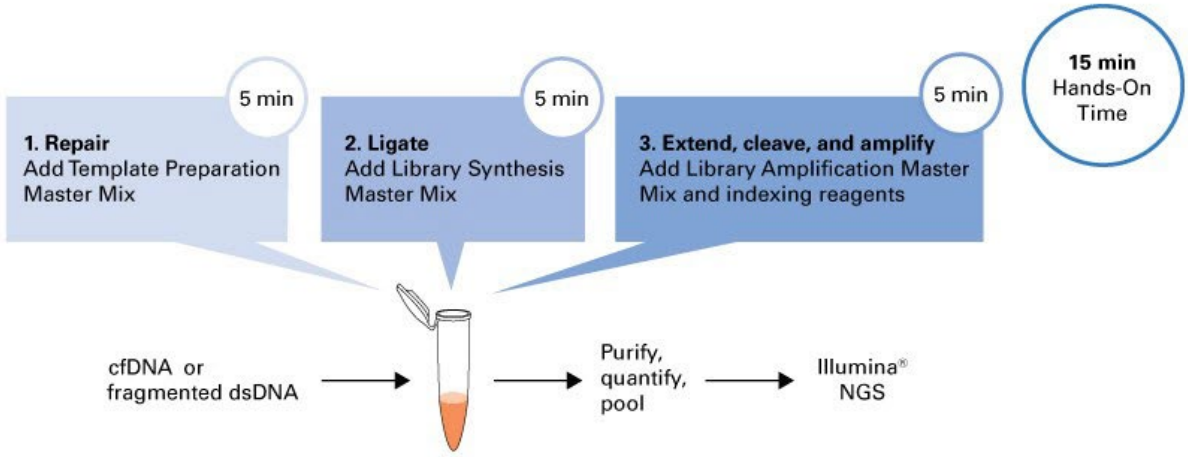

Figure 1. ThruPLEX Tag-Seq FLEX single-tube library preparation workflow. The ThruPLEX Tag-Seq FLEX workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify or transfer the sample material.

B. Principle

The ThruPLEX Tag-Seq FLEX kit is based on our patented ThruPLEX technology (Figure 2). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, ThruPLEX uses stem-loop adapters to construct high-quality libraries in a fast and efficient workflow. In the first step, template preparation, DNA is repaired yielding molecules with blunt ends. In the next step, stem-loop adapters with blocked 5' ends are ligated with high efficiency to the 5' ends of the genomic DNA, leaving nicks at the 3' ends. 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 library preparation methods. 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.

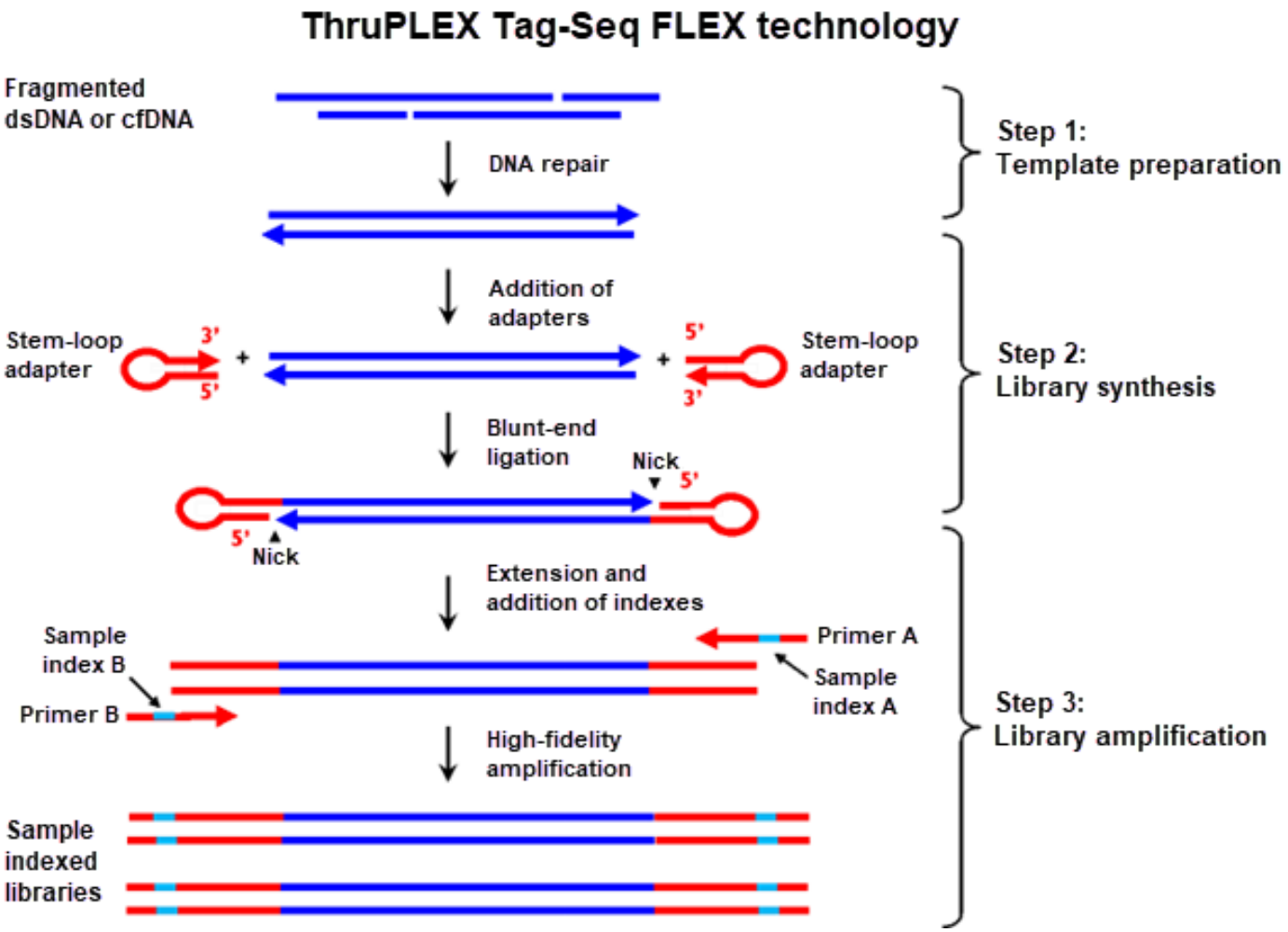

Figure 2. ThruPLEX Tag-Seq FLEX technology uses a three-step, single-tube reaction. It starts with fragmented double-stranded DNA or cfDNA (1 ng to 200 ng). Stem-loop adapters with molecular tags (not shown) are blunt-end ligated to repaired input DNA. These molecules are extended, then amplified to include barcodes using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX Tag-Seq FLEX 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 could lead to false positives. The three-step, single-tube library preparation

workflow (Figure 1) is the simplest in the industry—minimizing handling errors and 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 can impact the accuracy of results. The ThruPLEX Tag-Seq FLEX kit was designed to overcome these challenges through the incorporation of discrete unique molecular identifiers (UMI) in the ThruPLEX adapters, which tag DNA molecules with 144 distinct UMI combinations. The UMI enable discrimination of PCR and sequencing errors from true genomic variants—particularly helpful for detection of low-frequency mutations with high sensitivity and specificity—and performance of the sequencing libraries is highly reproducible between replicates, sequencing runs, and samples.

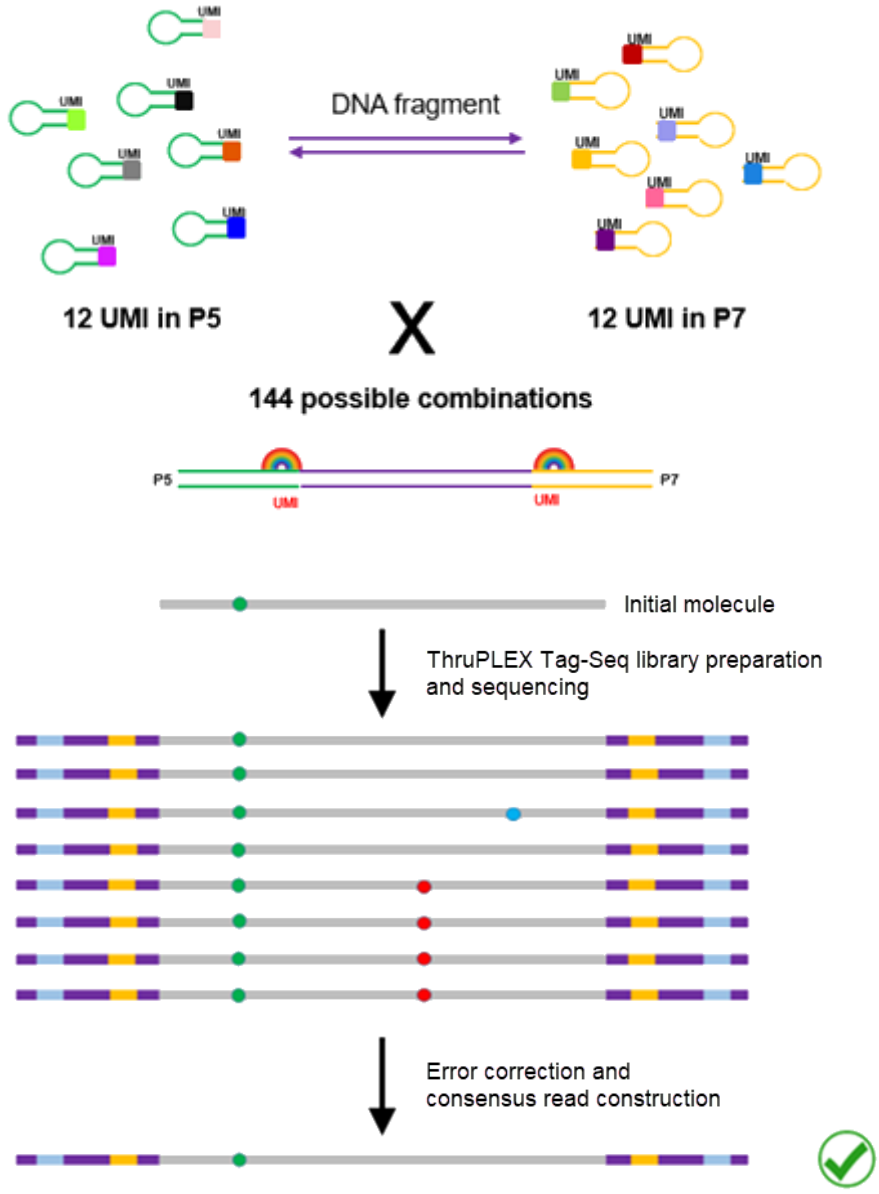

Figure 3. ThruPLEX Tag-Seq FLEX provides 144 possible combinations of discrete unique molecular identifiers (UMIs). ThruPLEX Tag-Seq FLEX is designed to remove the ambiguity in variant calling by reducing the false positive calls coming from DNA polymerase and sequencing errors. The seven-base UMIs are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

D. ThruPLEX Tag-Seq FLEX Workflow

The ThruPLEX Tag-Seq FLEX 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 FLEX workflow takes place in a single tube or well and is completed in about two hours.

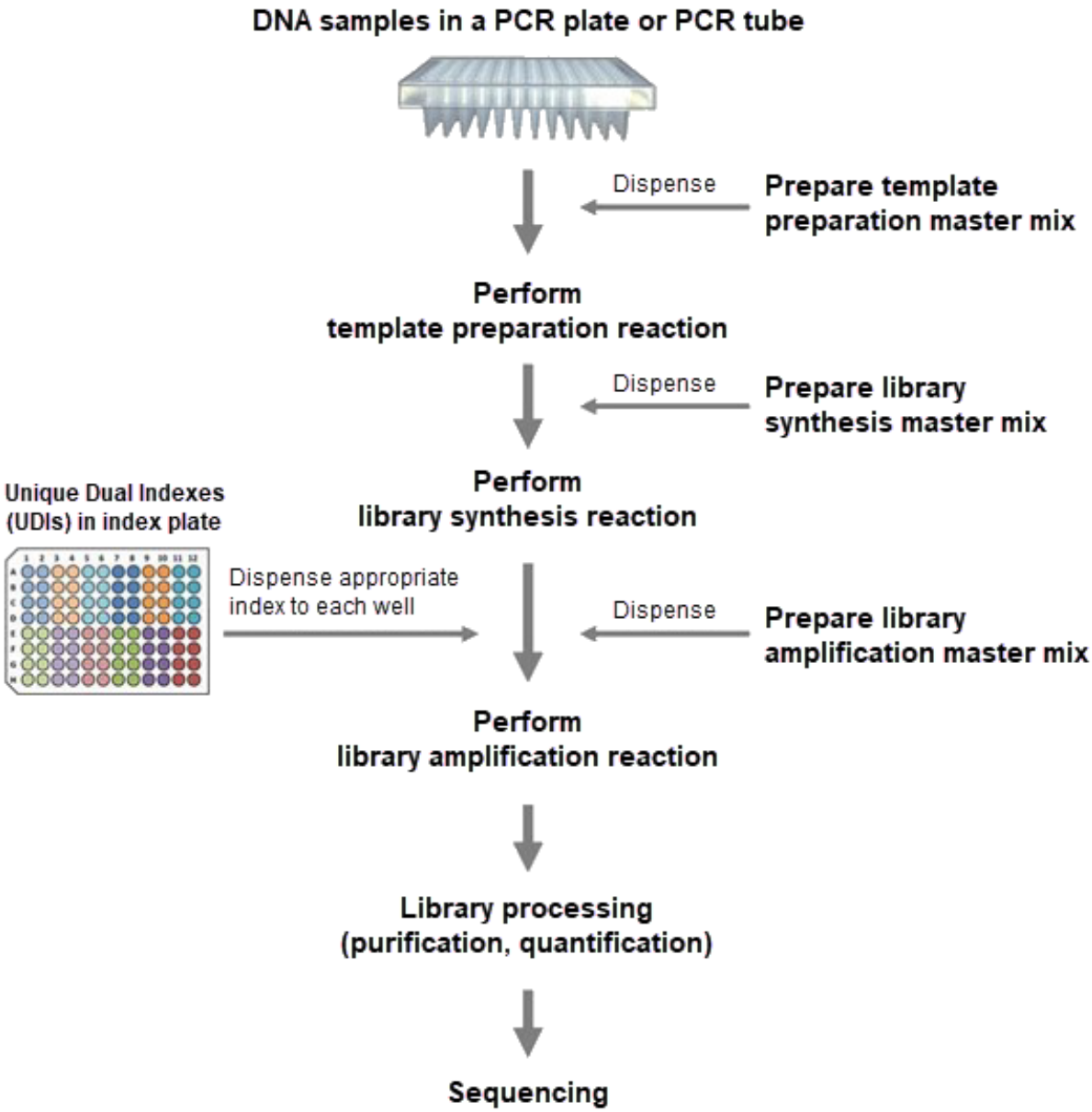

Figure 4. Overview of ThruPLEX Tag-Seq FLEX library preparation for Illumina NGS, starting with fragmented DNA.

II. List of Components

The ThruPLEX Tag-Seq FLEX 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 cycles**. Contents of the ThruPLEX Tag-Seq FLEX kit are not interchangeable with other Takara Bio products.

NOTE: Indexing primers are not included in the kit and need to be purchased separately (Unique Dual Index Kit, Takara Bio, Cat. Nos. 634752–634756).

Table 1. ThruPLEX Tag-Seq FLEX contents.

ThruPLEX Tag-Seq FLEX (Store at –20°C)	Cap color	R400734 (24 rxns)	R400735 (96 rxns)
Control Fragmented Human gDNA (5 ng/µl)	N/A	10 µl	10 µl
PBT1	Blue	140 µl	560 µl
PET1	Blue	30 µl	120 µl
SBT1	White	55 µl	220 µl
SET1	White	110 µl	440 µl
ABT1	Violet	1,250 µl	4 x 1,250 µl
AET1	Violet	55 µl	220 µl
Nuclease-Free Water	Clear	1,100 µl	1,100 µl

III. Additional Materials Required

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

- Unique Dual Index Kit (Cat. Nos. 634752–634756)
- Hot-lid PCR thermal cycler (real-time instrument optional)

  NOTE: See Thermal Cycler Considerations in Section IV.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.

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

IV. General Considerations

A. Sample Requirements

Table 2. 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	1 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 FLEX. 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 3. 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 1 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 a higher input amount of DNA.

Table 4. Estimated gene copies available for library preparation based on input amount and allele frequency.

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
1 ng	333	16	3	1

*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. ThruPLEX Tag-Seq FLEX 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.

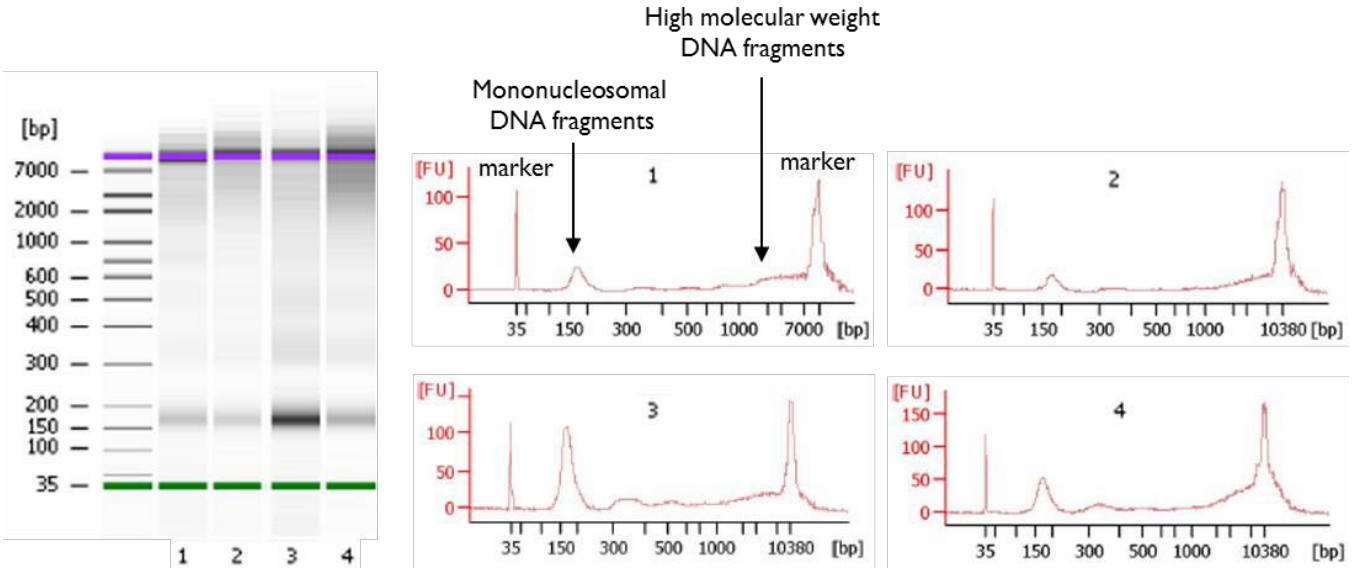

Figure 5. Fragment size distribution of cfDNA isolated from plasma. cfDNA from four different human plasma samples was isolated and analyzed using the Agilent Bioanalyzer. Distinct features of the isolated cfDNA are peaks (or bands) centered around 170 bp and higher molecular weight fragments.

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 the use of Unique Dual Index Kit indexing reagents (Cat. Nos. 634752–634756) with ThruPLEX Tag-Seq FLEX. These UDI kits are sold separately in 24-reaction or 96-reaction sizes and allow for multiplexing of up to 384 samples.

Indexing reagents consist of amplification primers containing Illumina-compatible indexes. Index sequences can be downloaded as .xlsx files at the Unique Dual Index Kit Product Page, under the Documents tab. Before starting the ThruPLEX Tag-Seq FLEX library preparation protocol (Section V.A), refer to Unique Dual Index Kit product documentation for information on index sequences, Index Plate handling instructions, and multiplexing and index-pooling guidelines.

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 FLEX library preparation protocol (Section V.A), create a Sample Sheet in the IEM to select and validate appropriate indexes to use in your experiments.

5. Target Enrichment

ThruPLEX Tag-Seq FLEX is compatible with major target enrichment products. ThruPLEX Tag-Seq FLEX 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 FLEX libraries to build consensus sequences. In general, detection of alleles present at lower frequencies requires sequencing at a higher depth. The prerequisite to a higher depth of sequencing is a high complexity library that yields more novel reads (non-saturation). ThruPLEX Tag-Seq FLEX offers this quality in a consistent way for every sample.

Table 5. 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.

V. Protocols

A. Protocol: ThruPLEX Tag-Seq FLEX 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:** 1–200 ng fragmented DNA
   - **Samples:** Dispense 30 µl of cfDNA or fragmented doubled-stranded DNA (33 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:

5 µl	PBT1 (Blue cap)
1 µl	PET1 (Blue cap)
6 µl	**Total volume/reaction**

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:

30 µl	Sample or control (fragmented DNA input)
6 µl	Template preparation master mix
36 µl	**Total volume/reaction**

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:

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:

2 µl	SBT1 (White cap)
4 µl	SET1 (White cap)
6 µl	**Total volume/reaction**

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:

36 µl	Template preparation reaction product
6 µl	Library synthesis master mix
42 µl	**Total volume/reaction**

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:

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 Indexing Reagents (UDI plate) as described below:
   - Remove the Indexing Reagents from the freezer and thaw for ten min on the bench top
   - Spin the Indexing Reagents 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:

46 µl	ABT1 (Violet cap)
2 µl	AET1 (Violet cap)
5 µl	Nuclease-Free Water (Clear cap)
53 µl	**Total volume/reaction**

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 5 µl of the appropriate Indexing Reagent.

Library amplification reaction mixture:

42 µl	Library synthesis reaction product
53 µl	Library amplification master mix
5 µl	UDI
100 µl	**Total volume/reaction**

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.

72°C	3 min	**1**	**Extension**
85°C	2 min	**2**	
98°C	2 min	**3**	**Denaturation**
7–16 cycles*:		**4**	**Library Amplification**
98°C	20 sec		
65°C	75 sec		
68°C	5 min	**5**	**Final extension**
4°C	Hold	**6**	

*See Table 6, "▲Stage 4 amplification guide".

Table 6. ▲Stage 4 amplification guide.

Input DNA	Number of cycles required to generate 500–1,000 ng library
200 ng	7–8
100 ng	8–9
50 ng	9–10
10 ng	11–12
5 ng	12–13
1 ng	15–16

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 workstation 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. Place 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 FLEX Library Quantification and Quality Assessment

Quantification of the libraries can be performed using real-time PCR with a library quantification kit for Illumina NGS libraries, such as the Library Quantification Kit (Takara Bio, Cat. No. 638324), or by fluorescence detection-based methods, such as Qubit Fluorometer (Thermo Fisher Scientific) or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). 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 a 1 µl aliquot of this diluted sample onto a Bioanalyzer high-sensitivity DNA chip (Agilent Technologies, Cat. No. 5067-4626).

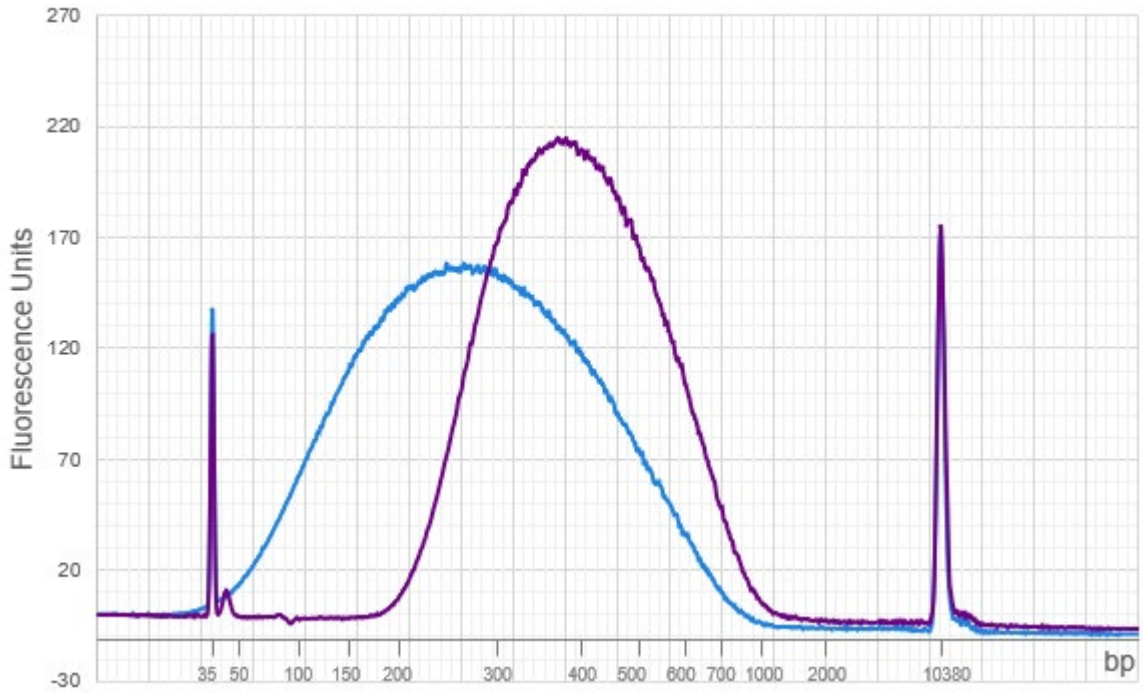

Figure 6. Bioanalyzer traces from 250 bp control Covaris-sheared genomic DNA before and after library preparation. The ThruPLEX Tag-Seq FLEX library (purple) was generated from ~250 bp sheared gDNA (blue).

Appendix A. Troubleshooting Guide

Table 7. Troubleshooting Guide for ThruPLEX Tag-Seq FLEX.

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 IV.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 >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.

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