Takara Bio USA

ThruPLEX® DNA-Seq FLEX User Manual

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(091524)

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I. Introduction

A. Overview

ThruPLEX DNA-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 250 pg–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 DNA-Seq FLEX libraries excel when combined with target enrichment and deliver high-quality sequencing results.

Pairing ThruPLEX DNA-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.

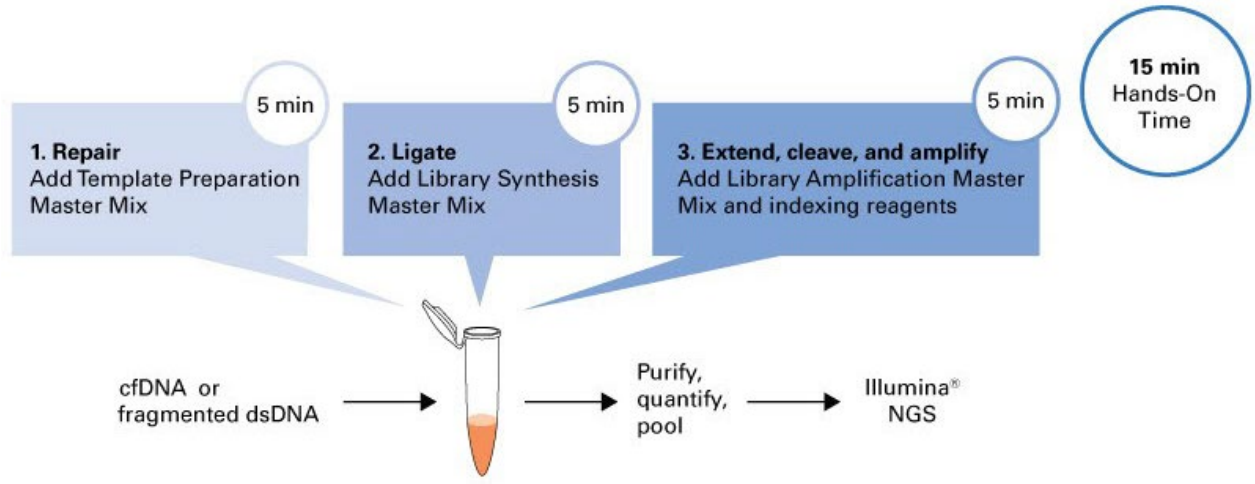

Figure 1. ThruPLEX DNA-Seq FLEX single-tube library preparation workflow. The ThruPLEX DNA-Seq FLEX workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify and transfer the sample material.

B. Principle

ThruPLEX DNA-Seq FLEX is based on our patented ThruPLEX technology (Figure 2). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, ThruPLEX technology 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.

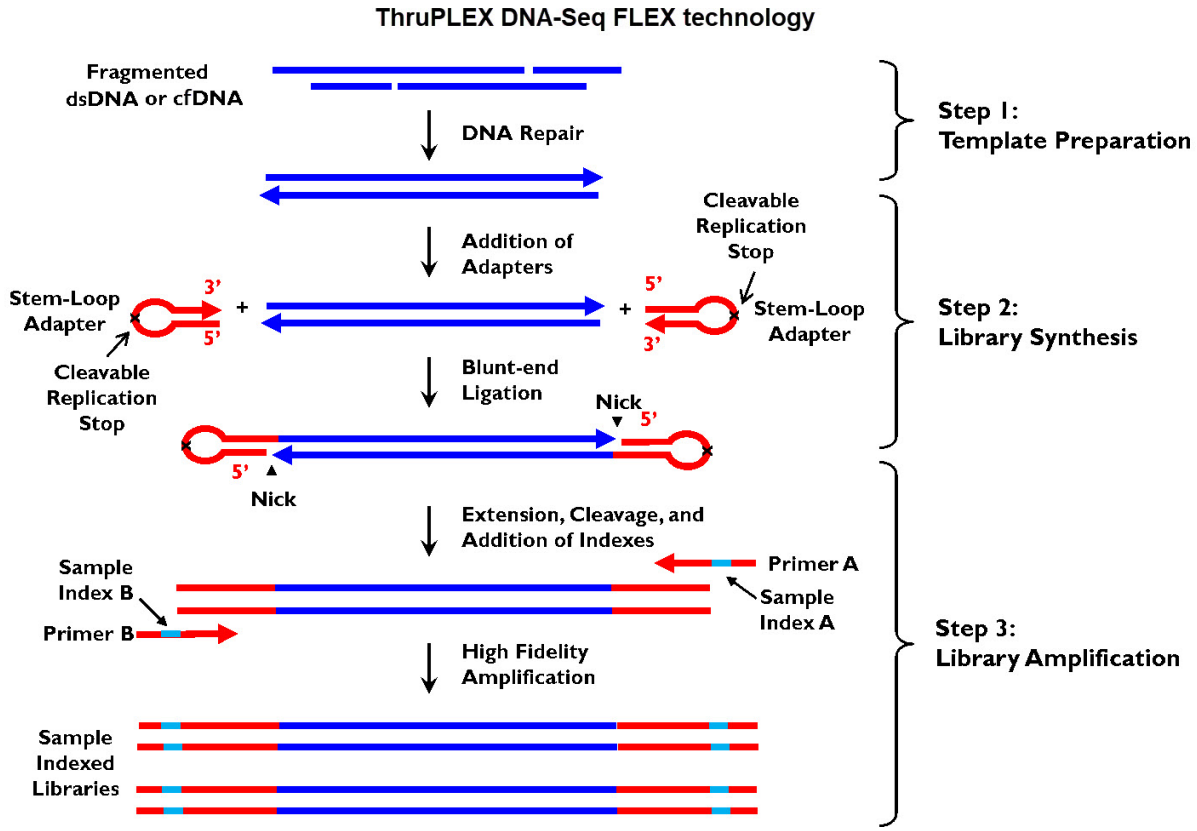

Figure 2. ThruPLEX DNA-Seq FLEX technology uses a three-step, single-tube reaction. It starts with fragmented double-stranded DNA or cfDNA (250 pg to 200 ng). Stem-loop adapters are blunt-end ligated to repaired input DNA. These molecules are extended, then amplified to include barcodes using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX DNA-Seq FLEX Workflow

The ThruPLEX DNA-Seq FLEX workflow is highly streamlined (Figure 3) 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, 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 FLEX workflow takes place in a single tube or well and is completed in about two hours.

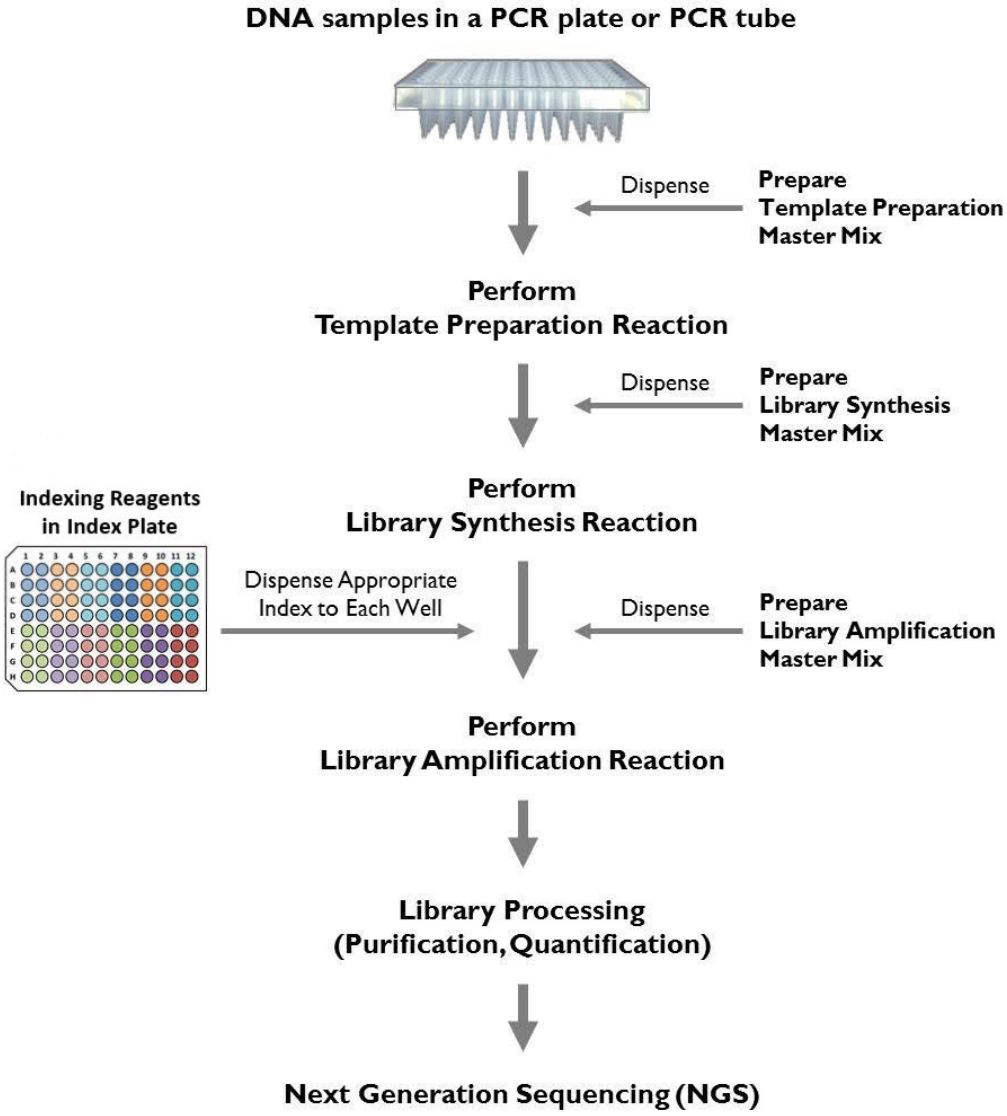

Figure 3. Overview of ThruPLEX DNA-Seq FLEX library preparation for Illumina NGS, starting with fragmented DNA.

II. List of Components

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 DNA-Seq FLEX components.

ThruPLEX DNA-Seq FLEX (Store at –20°C)	Cap color	R400737 (24 rxns)	R400736 (96 rxns)
Control Fragmented Human gDNA (5 ng/µl)	N/A	10 µl	10 µl
PBD1	Blue	140 µl	550 µl
PED1	Blue	30 µl	110 µl
SBD1	White	30 µl	110 µl
SED1	White	110 µl	440 µl
ABD1	Amber	1,160 µl	4 x 1,160 µl
AED1	Violet	60 µl	220 µl
Nuclease-Free Water	Clear	1,100 µl	1,100 µl

III. Additional Materials Required

- Unique Dual Index Kit (Takara Bio, 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
- PCR plate seals (if using plates)
- Single-channel pipette: 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 Qubit, for library quantification
- 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

- 20X fluorescent dye, such as EvaGreen, in water (Biotium, Cat. No. 31000-T)
- Calibration Dye (such as Bio-Rad Laboratories, Cat. No. 170-8780)
- Reference Dye (if required by real time instrument)
- 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

	DNA Sample Requirements
Nucleic Acid	Fragmented double-stranded DNA or cfDNA
Source	Cells, plasma, urine, other biofluids, FFPE, tissues, fresh tissues, frozen tissues
Type	Mechanically sheared; enzymatically fragmented; low-molecular-weight cell-free DNA
Molecular Size	<1,000 bp
Input Amount	250 pg 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 DNA-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 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 250 pg 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
250 pg	83	4	0	0

*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 TE (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 between 150 and 500 bp. ThruPLEX DNA-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

cfDNA isolated from plasma samples contains both high and low molecular weight DNA fragments (Figure 4). 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.

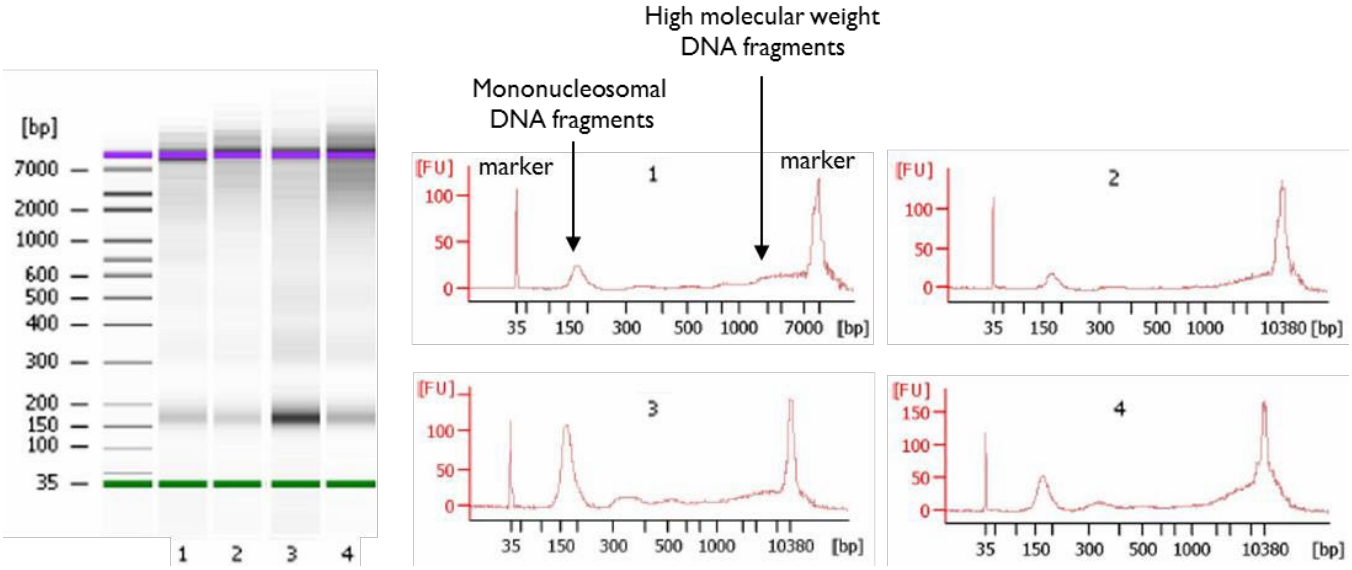

Figure 4. Fragment size distribution of cfDNA isolated from plasma. cfDNA from four different human plasma samples was isolated and analyzed using the Agilent Bioanalyzer. Distinct features of the isolated cfDNA are a peak (or band) centered around 170 bp and higher molecular weight fragments.

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 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.

Monitoring amplification during the library amplification reaction

Amplification can be monitored using a real-time thermal cycler with the addition of fluorescent dyes (not provided with the kit, see Optional Materials in Section III) to the reaction (Figure 4). If a regular thermal cycler is used instead, there is no need to add the dyes; use an appropriate amount of nuclease-free water to prepare the library amplification master mix. In the absence of real-time monitoring, library amplification can be analyzed by gel or by analysis of an aliquot of the library using the Agilent Bioanalyzer (see "Library Quantification", Section V.B.3).

Depending on the real-time instrument used, select an appropriate calibration dye and mix with EvaGreen dye to prepare the dye mix (see Library Amplification Step, Section V.A.3). For some real-time instruments, calibration dye may not be needed; please refer to the real-time thermal cycler instrument's manual.

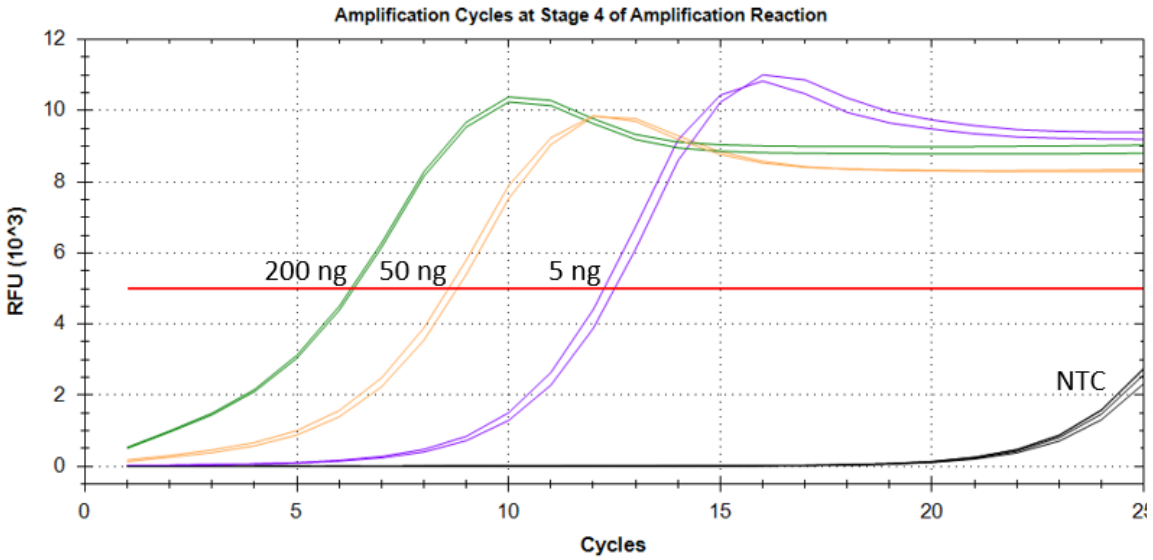

Figure 5. Example of real-time analysis of library amplification using ThruPLEX DNA-Seq FLEX. A typical real-time amplification analysis of libraries prepared with ThruPLEX DNA-Seq FLEX using 200 ng, 50 ng, or 5 ng of the provided positive control DNA compared to a No Template Control (NTC). Results were obtained using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with EvaGreen as the dye for detection and fluorescein as the calibration dye. The red line marks the midpoint of the linear phase of the amplification curves and is used to determine the optimal number of amplification cycles at Stage 4 of the library amplification reaction (Section V.A). It is recommended to stay within one cycle above or below the optimal number of cycles. For example, for a 5-ng input, the optimal number of amplification cycles is 12 ±1 cycles or 11–13 cycles. The Relative Fluorescence Unit (RFU) values on the y-axis may vary based on the instrument used.

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 D master mix and library amplification D 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 DNA-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 DNA-Seq FLEX Library Preparation Protocol (Section V.A), refer to Unique Dual Index Kit product documentation for information on index sequences, plate handling instructions, and multiplexing and index-pooling guidelines.
Indexing reagents are predispensed and sealed in a linear barcoded 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 DNA-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 DNA-Seq FLEX is compatible with major target enrichment products. ThruPLEX DNA-Seq FLEX 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 Material Safety Data Sheets (MSDS) available online at takarabio.com.

V. Protocols

A. Protocol: ThruPLEX DNA-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:
   - **Samples:** Dispense 30 µl of fragmented doubled-stranded DNA, or cfDNA 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 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 D master mix as described in the table below for the desired number of reactions, plus 10% excess to allow for pipetting losses. Mix thoroughly with a pipette. Keep on ice until used.

Template preparation D master mix:

5 µl	PBD1 (Blue cap)
1 µl	PED1 (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 D master mix.

Template preparation reaction mixture:

30 µl	Sample or control
6 µl	Template preparation D master mix
36 µl	**Total volume/reaction**

Mix thoroughly at least 10 times with a pipette set to 25 µl. Avoid introduction of air bubbles.

4. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
5. Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each well.
6. 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

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

NOTE: Following the template preparation step, 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, plus 10% excess to allow for pipetting losses. Mix thoroughly with a pipette. Keep on ice until used.

Library synthesis D master mix:

1 µl	SBD1 (White cap)
4 µl	SED1 (White cap)
5 µl	**Total volume/reaction**

2. Remove the seal on the plate or open the tube(s).
3. Assemble the library synthesis reaction mixture as shown below. To each well or tube, add 5 µl of the library synthesis D master mix.

Library synthesis reaction mixture:

36 µl	Template Preparation Reaction Product
5 µl	Library synthesis D master mix
41 µl	**Total volume/reaction**

Mix thoroughly at least 10 times with a pipette set to 25 µl.

4. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
5. Centrifuge briefly to collect the contents at the bottom of each well or tube.

6. 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 below:

30°C	40 min
4°C	Hold for ≤30 min

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

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

3. Library Amplification

Multiple stages occur during the library amplification reaction (see Step 10, below). During Stage 1 and Stage 2, the 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 and thermal cycler used. Use the table below (repeated in Table 5) 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**
200 ng	7–8
100 ng	8–9
50 ng	9–10
5 ng	12–13
1 ng	15–16
0.5 ng	16–17
0.25 ng	17–18

- **Optimization experiment:** Performing an optimization experiment to identify the appropriate number of PCR cycles needed 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 by constructing PCR curves and identifying the midpoint of the linear phase as illustrated in Figure 4. Use the optimal amplification cycle number in the actual experiment for sequencing.
- **Yield:** The amount of amplified library can range from 100 ng–1 µg depending upon many variables including sample type, fragmentation size, and thermal cycler used. When starting with Covaris-fragmented reference DNA with an average size of 200 bp and following the recommended number of amplification cycles, the typical yields range from 500–1,000 ng.

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

For this step, you will need the following components: ABD1 (amber cap), AED1 (violet cap), Nuclease-Free Water (clear cap), fluorescent dyes (optional), and a Unique Dual Index Kit

NOTES:
- It is critical to handle the UDIs following the provided instructions in the Unique Dual Index Kit Protocol-At-A-Glance to avoid cross contamination of indexes.
- No more than four freeze/thaw cycles are recommended.

1. Remove the UDI kit from freezer and thaw for ten min on the bench top.
2. Spin the UDI plate in a tabletop centrifuge to collect contents at the bottom of the well.
3. Confirm ABD1 is fully thawed and thoroughly homogenized by heating briefly at 25°C and vortexing vigorously for 30 sec.
4. Prepare library amplification D master mix as described in the table below for the desired number of reactions, plus 10% excess to allow for pipetting losses. Mix thoroughly with a pipette. Keep on ice until used.

Library amplification D master mix:

42 µl	ABD1
2 µl	AED1
10 µl	Nuclease-Free Water (plus fluorescent dyes*)
54 µl	**Total volume/reaction**

NOTES:
- **If monitoring in real-time:** Fluorescence dyes* (for detection and optical calibration) are added when monitoring amplification in real time during cycling. Please refer to the real-time PCR instrument's user manual for calibration dye recommendations. The volume of detection and calibration dyes plus nuclease-free water should not exceed 10 µl.
- **If not monitoring in real-time:** If a regular thermal cycler is used, there is no need to add the dyes; use 10 µl of nuclease-free water per reaction in the library amplification D master mix.

5. Remove the seal on the PCR plate or open the tube(s).
6. Add 54 µl of the library amplification D master mix to each well or tube.
7. Add 5 µl of the appropriate UDI to each well or tube.

Library synthesis reaction mixture:

41 µl	Library synthesis reaction product
54 µl	Library amplification D master mix
5 µl	UDI
100 µl	**Total volume/reaction**

NOTES:
- Make sure that the barcode label on the long side of the index plate is facing you.
- Thoroughly wipe the plate seal with 70% ethanol and allow it to dry to prevent cross-contamination.

8. Mix thoroughly a minimum of 10 times with a pipette set to 70 µl. Avoid introducing excessive air bubbles.
9. 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.

   NOTE: Use optical sealing film or caps if a real-time thermal cycler is used.

10. Return the plate or tube(s) to the thermal cycler with heated lid set to 101–105°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 & cleavage**
85°C	2 min	**2**	
98°C	2 min	**3**	**Denaturation**
7–18 cycles*:		**4**	**Library Amplification**
98°C	20 sec		
68°C†	75 sec		
68°C	5 min	**5**	**Final extension**
4°C	Hold	**6**	

*See Table 5, "▲Stage 4 amplification guide".
†If monitoring amplification in real-time, acquire fluorescence data at this step.

Table 5. ▲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
5 ng	12–13
1 ng	15–16
0.5 ng	16–17
0.25 ng	17–18

11. 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 processed for sequencing immediately or stored frozen at –20°C for later processing. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to Section V.B.

B. Library Processing for Illumina Next-Generation Sequencing

1. Overview

This section contains guidelines for processing ThruPLEX DNA-Seq FLEX libraries for Illumina NGS. In some cases, recommended protocols are listed (Library Purification by AMPure XP Beads) 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 the amplification. needed. 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 and quantification, and quality are described in the following sections.

2. Library Purification by AMPure XP Beads

AMPure XP is the recommended method of library purification. Do not use QIAquick cleanup or other silica-based filters for purification as this will result in incomplete removal of primers.
The ratio of AMPure XP 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 protocols for DNA purification.

For this step, you will need the following components: AMPure XP beads, Magnetic rack for 200 µl strip tubes, freshly prepared 80% (v/v) ethanol, and TE buffer (pH 8.0)

NOTES:
- It is important to bring all the samples and reagents to be used to room temperature.
- Always use freshly prepared 80% (v/v) ethanol for Step 3 and Step 4 below.
- Resuspend the AMPure XP reagent 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 with 100-µl 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 lX 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, 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, using fluorescence detection-based methods, Qubit Fluorometer (Thermo Fisher Scientific), or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies), or using 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 a 1-µl aliquot of this diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, Cat. No. 5067-4626). Libraries prepared using ThruPLEX DNA-Seq FLEX result in a size distribution of library fragments that is dependent on the input DNA fragment size (Figure 6).

NOTE: The adapters added during the ThruPLEX DNA-Seq FLEX library preparation process result in an approximately 140 base pair increase in the size of each library.

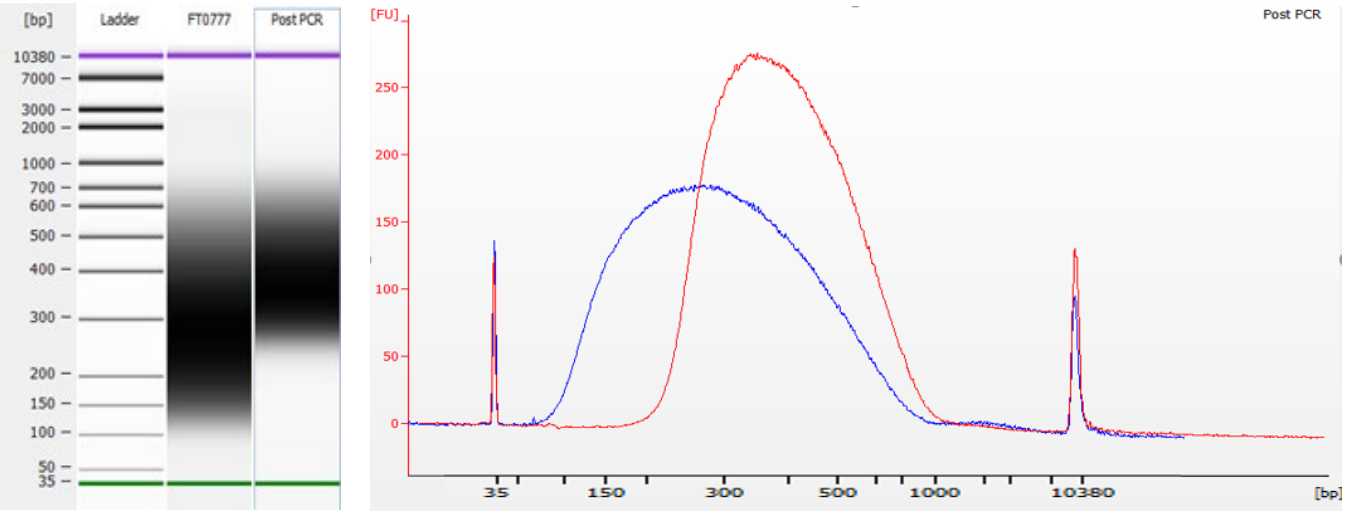

Figure 6. Bioanalyzer analysis of libraries prepared using ThruPLEX DNA-Seq FLEX. Libraries were prepared from 5-ng of the provided positive control DNA (blue trace) using the ThruPLEX DNA-Seq FLEX. Post library amplification, libraries were purified following the AMPure XP protocol (V.B.2). An aliquot of purified library was diluted to 5 ng/µl in TE buffer, and 1 µl of this diluted sample (red trace) was loaded onto a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies).

Appendix A. Troubleshooting Guide

Table 6. Troubleshooting guide for ThruPLEX DNA-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.

Problem	Potential Cause	Suggested Solutions
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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This document has been reviewed and approved by the Quality Department.