Takara Bio USA

ThruPLEX® DNA-Seq FLEX EF User Manual

Cat. Nos. 635100 & 635101
(021125)

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I. Introduction

A. Overview

ThruPLEX DNA-Seq FLEX EF (Cat. Nos. 635100 & 635101) is designed to provide up to 384 indexed libraries for higher multiplexing capabilities on Illumina® NGS platforms. ThruPLEX DNA-Seq FLEX EF 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 100 pg–200 ng double-stranded DNA (dsDNA) are required for library preparation. The entire three-step workflow takes place in a single tube or well, in about two hours (Figure 1). No intermediate purification steps or sample transfers are necessary, thus preventing handling errors and loss of valuable samples. With high library diversity, ThruPLEX DNA-Seq FLEX EF libraries excel when combined with target enrichment to deliver high-quality sequencing results.

The ThruPLEX DNA-Seq FLEX EF Kit combines the ThruPLEX FLEX Enzymatic Fragmentation Module (Cat. No. R400780 & R400781) and ThruPLEX DNA-Seq FLEX (Cat. No. R400736 & R400737). The ThruPLEX FLEX Enzymatic Fragmentation Module is designed to perform size-tunable enzymatic fragmentation in tandem with the ThruPLEX DNA-Seq FLEX repair step. This eliminates additional time for fragmentation, such as mechanical fragmentation, or separate enzymatic fragmentation modules.

Pairing ThruPLEX DNA-Seq FLEX EF with unique dual indexes (UDIs) allows for multiplexing 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 other enrichment techniques.

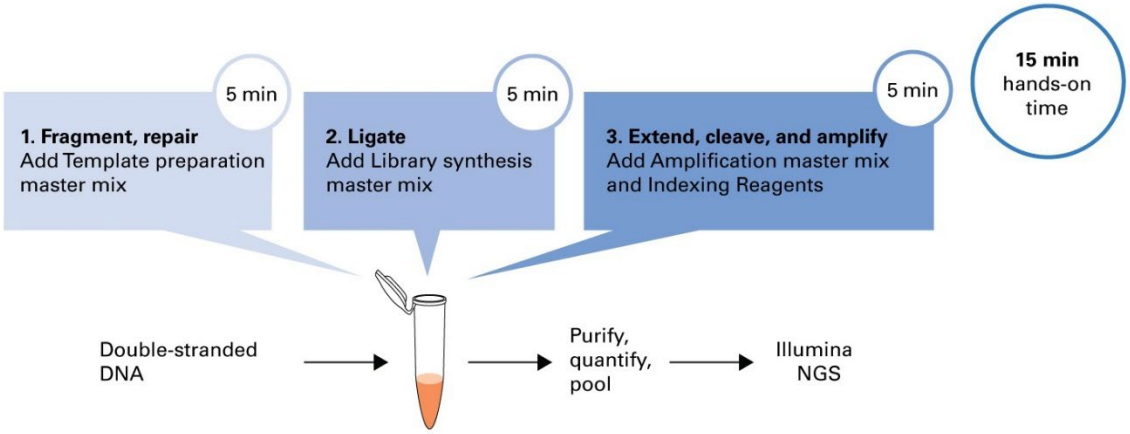

Figure 1. ThruPLEX DNA-Seq FLEX EF single-tube library preparation workflow. The ThruPLEX DNA-Seq FLEX EF workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify and transfer the sample material.

B. Principle

ThruPLEX DNA-Seq FLEX EF is based on our patented ThruPLEX technology (Figure 2). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, ThruPLEX FLEX uses stem-loop adapters to construct high-quality libraries in a fast and efficient workflow. In the first step, template preparation, the DNA is fragmented and repaired to leave blunt ends. In the next step, stem-loop adapters

with blocked 5' ends are ligated with high efficiency to the 5' end of the genomic DNA, leaving a nick at the 3' end. The adapters cannot ligate to each other and do not have single-stranded tails, preventing the nonspecific background typically found with many other NGS preparations. In the final step, the 3' ends of genomic DNA are extended to complete library synthesis, and Illumina-compatible indexes are added through high-fidelity amplification. Any remaining free adapters are destroyed. Hands-on time and risk of contamination are minimized by using a single tube and eliminating intermediate purification steps.

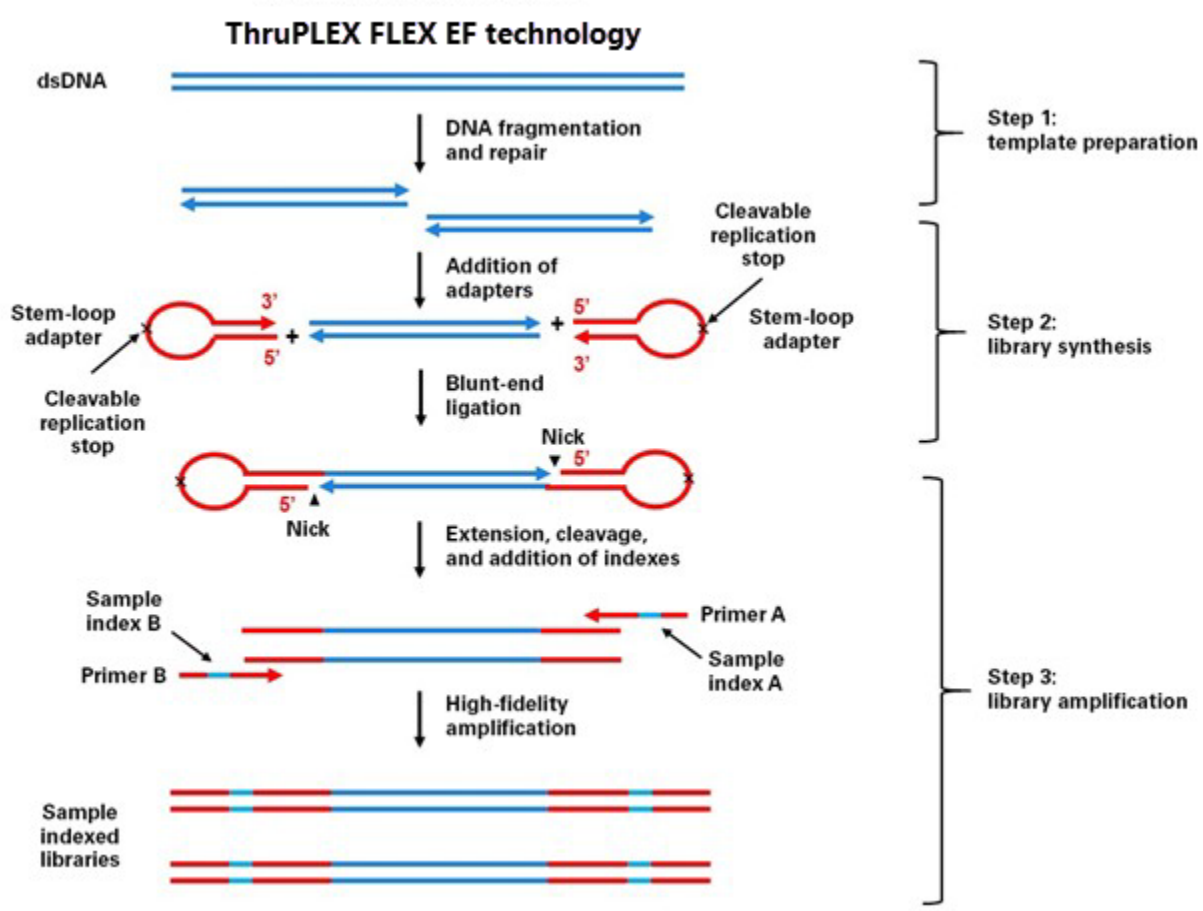

Figure 2. ThruPLEX FLEX EF technology uses a three-step, single-tube reaction. The workflow starts with dsDNA (100 pg–200 ng). Stem-loop adapters are blunt-end ligated to repaired input DNA. These molecules are extended, then amplified to include barcodes using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX DNA-Seq FLEX EF Workflow

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

- **Template preparation** for fragmentation and end-repair of the dsDNA 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 EF workflow takes place in a single tube or well and is completed in about two hours (Figure 3).

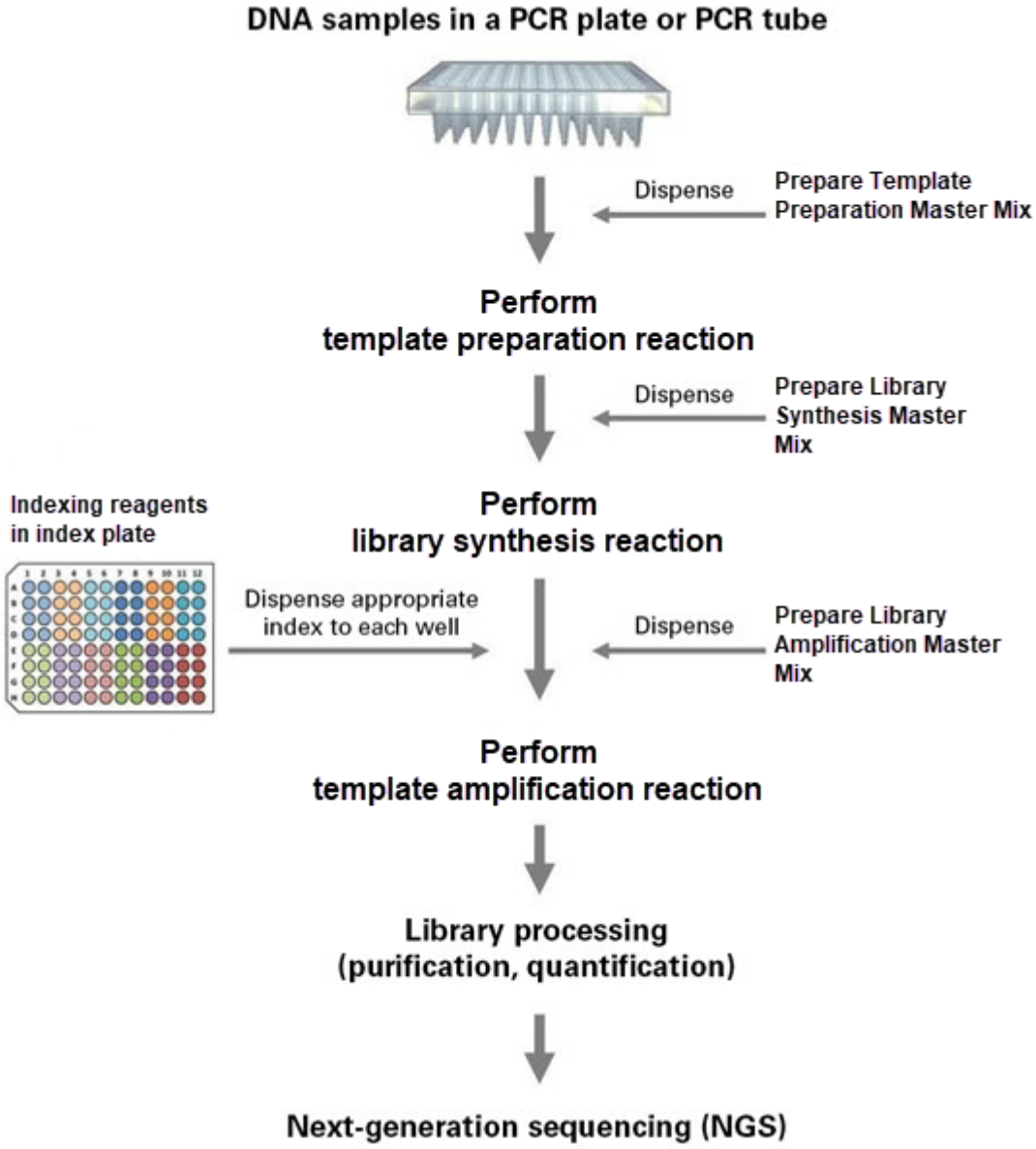

Figure 3. Overview of ThruPLEX DNA-Seq FLEX EF library preparation for Illumina NGS.

II. List of Components

ThruPLEX DNA-Seq FLEX EF kits contain sufficient reagents to prepare 24 (Cat. No. 635100) or 96 (Cat. No. 635101) reactions.

IMPORTANT: Please do not make any substitutions. The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.

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

Table 1. ThruPLEX DNA-Seq FLEX EF Kit contents.

ThruPLEX DNA-Seq FLEX EF	Cap color	635100 (24 rxns)	635101 (96 rxns)
ThruPLEX DNA-Seq FLEX (Store at –20°C)		**R400737***	**R400736***
Control Fragmented Human gDNA (5 ng/µl)	–	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 tube	1,160 µl	4 x 1,160 µl
AED1	Violet	60 µl	220 µl
Nuclease-Free Water	Clear	1,100 µl	1,100 µl
ThruPLEX FLEX Enzymatic Fragmentation Module (Store at –20°C)		**R400780***	**R400781***
Control Human gDNA (5 ng/µl)	Red	10 µl	10 µl
10X PDF1	Light blue	96 µl	384 µl
PBF1	Yellow	120 µl	480 µl
PEF1	Green	60 µl	240 µl
Nuclease-Free Water	White	1,000 µl	4 x 1,000 µl

*Not sold separately.

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 pipettes: 10 µl, 20 µl, and 200 µl

- Multi-channel pipettes: 20 µl and 200 µl
- Low-binding filter pipette tips: 10 µl, 20 µl, and 200 µl
- Low-binding aerosol barrier tips
- Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0)
- 80% (v/v) ethanol: freshly made for each experiment
- Magnetic separator, such as SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
- Fluorometer, such as Thermo Fisher Scientific Qubit, for library quantification
- NucleoMag NGS Clean-up and Size Select (Takara Bio, Cat. Nos. 744970.5, 744970.50 & 744970.500) or Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880)

NOTES: NucleoMag NGS Clean-up and Size Select and Agencourt AMPure XP beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads upon receipt, then refrigerating the aliquots.

– Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min).

– Aliquoting also minimizes the chances of bead contamination.

Optional Materials

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

IV. General Considerations

A. Sample Requirements

Table 2. DNA sample requirements.

DNA sample requirements	
Nucleic acid	Double-stranded DNA
Source	Cells, fresh tissues, frozen tissues, microbes
Input amount	100 pg–200 ng
Input volume	30 µl
Input buffer	≤10 mM Tris, ≤0.1 mM EDTA

1. General Guidelines

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

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

2. DNA Isolation

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

Table 3. Recommended DNA purification kits.

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

3. Input DNA Amount

The recommended input amount is 100 pg–200 ng of DNA quantified by Qubit Fluorometer or equivalent methods. Use an appropriate input amount of DNA to ensure sufficient variant copies are available to achieve the desirable detection sensitivity. In general, detection of alleles present at low frequencies requires a higher input amount of DNA.

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

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
100 pg	33	1	0	0

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

4. Input Volume

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

5. Input Buffer

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

6. Fragment Size

The ThruPLEX FLEX Enzymatic Fragmentation Module provides a protocol optimal for DNA fragmentation of larger fragments down to 300–450 bp. ThruPLEX DNA-Seq FLEX EF is a ligation-based technology; ligated adapters result in an approximately 140 bp size increase of each DNA template fragment. Library molecules with shorter inserts (200–300 bp) tend to cluster and amplify more efficiently on Illumina flow cells. Depending on the application and requirements, the solid-phase reversible immobilization purification step following library amplification (Section V.A.3) can be replaced with a size-selection step to remove unwanted fragments.

7. Positive and Negative Controls

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

B. General Recommendations

1. Thermal Cycler Considerations

- **Heated lid requirements**
  Use a thermal cycler equipped with a heated lid that can accommodate 100 µl reaction volumes. Set the temperature of the heated lid to 101°C–105°C to avoid sample evaporation during incubation and cycling.
- **Thermal cycler ramp rates**
  We recommend a ramp rate of 3°C/s–5°C/s; higher ramp rates are not recommended and could impact the quality of the library.

2. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared fresh at each workflow step, based on the number of reactions to be performed. The following steps are general guidelines; refer to the individual protocol steps for specific details on each mix.

IMPORTANT: Prepare ~10% excess of each master mix to allow for pipetting losses.

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

The Library Synthesis Master Mix and Library Amplification Master Mix can be prepared during the last 15 min of the previous step's cycling protocol and kept on ice until use.

3. Unique Dual Index Kits

We support the use of Unique Dual Index Kit indexing reagents (Cat. Nos. 634752–634756) with ThruPLEX DNA-Seq FLEX EF. These UDI kits are sold separately in 24-reaction (Cat. No. 634756) or 96-reaction (Cat. Nos. 634752–634755) 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] option for each catalog product.

Before starting the ThruPLEX FLEX EF library preparation protocol (Section V.A), refer to the 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

Install the latest version (v1.18.1 or later) of the Illumina Experiment Manager (IEM). Prior to starting the ThruPLEX DNA-Seq FLEX EF library preparation protocol (Section V.A), create a Sample Sheet in the IEM to select and validate appropriate indexes to use in your experiments. Refer to the Appendix for guidelines on using the IEM to validate your index combinations.

5. Target Enrichment

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

C. Safety Guidelines

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

V. Protocols

A. Protocol: ThruPLEX DNA-Seq FLEX EF Library Preparation

1. Template Preparation

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

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

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

Table 5. Enzyme dilution guide.

	Input DNA >50 ng		1–50 ng Input DNA		Input DNA <1 ng	
Reagent	**300 bp**	**450 bp**	**300 bp**	**450 bp**	**300 bp**	**450 bp**
1X PDF1	16 µl	25 µl	20 µl	30 µl	24 µl	36 µl
PEF1	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl

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

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

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

2. Prepare a 1X PDF1 mix by diluting 10X PDF1 (light blue cap) in a 1:10 ratio using Nuclease-Free Water for the desired number of reactions and fragment size (Section V.A.3, Table 6). Mix by vortexing. Store on ice.
3. On ice and using the previously determined optimal concentration from the enzyme dilution guide (Table 5), prepare the Fragmentation Enzyme Dilution as described below for the desired number of reactions and fragment size. Mix thoroughly by pipette. Store on ice.

   Fragmentation Enzyme Dilution:

Reagent	Cap color	Volume/reaction
1X PDF1 (from Step 2)	-	*X* µl*
PEF1	Green	2 µl

   *Where *X* is the value determined by Table 5

4. On ice, prepare Template Preparation D Master Mix as described below for the desired number of reactions, plus 10% of the total reaction mix volume. Mix thoroughly with a pipette.

   Template Preparation D Master Mix:

4 µl	PBF1 (green cap)
1 µl	PED1 (blue cap)
1 µl	Fragmentation Enzyme Dilution (from Step 3)
6 µl	**Total volume per reaction**

5. On ice, assemble the Template Preparation Reactions Mixture as shown in the table below. To each 30 µl sample from Step 1 above, add 6 µl of the Template Preparation D Master Mix.

   Template Preparation Reactions Mixture:

30 µl	Sample or control (from Step 1)
6 µl	Template Preparation D Master Mix (from Step 4)
36 µl	**Total volume per reaction**

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

6. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
7. Centrifuge briefly to collect reaction contents at the bottom of each well. Keep sample tube on ice until the temperature of the thermal cycler reaches 22°C.
8. Place the plate or tube(s) in a thermal cycler with a heated lid set to 101°C–105°C. Perform the template preparation reaction using the conditions below:

22°C	25 min
70°C	20 min
4°C	Hold for ≤2 hr

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

NOT A SAFE STOPPING POINT: Continue to the next step in the same plate or tube(s).

2. Library Synthesis

1. Prepare a Library Synthesis D Master Mix as described in the table below for the desired number of reactions, plus 10% of the total reaction mix volume. 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 per reaction**

2. Remove the seal on the plate or open the tube(s) resulting from the template preparation protocol (previous section).
3. To each well or tube, add 5 µl of the Library Synthesis D Master Mix.

36 µl	Template preparation reaction product (Section V.A.1, Step 9)
5 µl	Library Synthesis D Master Mix (from Step 1)
41 µl	**Total volume per 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 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 ≤2 hr

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

NOT A SAFE STOPPING POINT: Continue to the next step in the same plate or tube(s), maintained at 4°C.

3. Library Amplification

Multiple stages occur during library amplification (see Table 7). During Stage 1 and Stage 2, stem-loop adapters are extended and cleaved. **Proper programming of the thermal cycler is critical for these first two steps to be completed correctly, with no denaturation step occurring until Stage 3.** Illumina-compatible indexes are incorporated into the template library in Stage 4 and the resulting template is amplified; the number of cycles required at this stage is dependent on the amount of input DNA used. In Stage 5, a final extension of the libraries occurs. Samples are cooled to 4°C in Stage 6, after which they are pooled and purified or stored at –20°C.

- **Selection of the optimal number of cycles for library amplification (Stage 4):** The number of PCR cycles required at Stage 4 of the library amplification reaction is dependent on the amount of input DNA, fragmentation size, and thermal cycler used. Use Table 6 as a guide for selecting the number of PCR cycles.

Table 6. Stage 4 library amplification guide. The table documents the number of cycles required to generate a 500–1,000-ng library based on the amount of input DNA and the insert size.

Input DNA	300 bp insert size	450 bp insert size
200 ng	7–8	8–9
100 ng	8–9	9–10
50 ng	9–10	10–11
5 ng	12–13	13–14
1 ng	15–16	16–17
0.5 ng	16–17	17–18
0.25 ng	17–18	18–19
0.1 ng	19–20	20–21

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

NOTES:

– Overamplification could result in a higher rate of PCR duplicates in the library.

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

1. Prepare the Unique Dual Index Kit(s) being used by removing them from freezer storage. Thaw for 10 min on the bench top.
2. While the UDIs are thawing, confirm ABD1 (amber tube) is fully thawed and thoroughly homogenized by heating briefly at 25°C and vortexing vigorously for 30 sec.

3. Prepare Library Amplification D Master Mix as described in the table below for the desired number of reactions, plus 10% of the total reaction mix volume. Mix thoroughly with a pipette. Keep on ice until used.

Library Amplification D Master Mix:

42 µl	ABD1 (amber tube)
2 µl	AED1 (violet cap)
10 µl	Nuclease Free Water (clear cap)
54 µl	**Total volume per reaction**

4. After the UDIs have thawed, spin the plate in a benchtop centrifuge to collect contents at the bottom of the wells.
5. Remove the seal on the PCR plate or open the tube(s) resulting from the library synthesis protocol (previous section).
6. To each well or tube, add 54 µl of the Library Amplification D Master Mix and 5 µl of the appropriate UDI in the order shown below.

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

41 µl	Library synthesis reaction product (Section V.A.2, Step 7)
54 µl	Library Amplification D Master Mix (from Step 3)
5 µl	UDI
100 µl	**Total volume per reaction**

Mix thoroughly a minimum of 10 times with a pipette set to 70 µl. Avoid the introduction of excessive air bubbles.

7. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s) and centrifuge briefly to collect the contents at the bottom of each well or tube.
8. Return the plate or tube(s) to the thermal cycler with heated lid set to 101°C–105°C. Perform the library amplification reaction using the conditions in Table 7.

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

Table 7. Library amplification reaction thermal cycler program.

	Stage	Temperature	Time	# cycles
Extension & cleavage	1	72°C	3 min	1
	2	85°C	2 min	1
Denaturation	3	98°C	2 min	1
Library amplification	4	98°C 68°C	20 sec 75 sec	7–21*
Final extension	5	68°C	5 min	1
Hold	6	4°C	Hold	1

*See Stage 4 amplification guide, Table 6 or repeated on the next page.

Stage 4 amplification guide		
Input DNA	Number of cycles required to generate a 500–1,000-ng library	
	300 bp insert size	450 bp insert size
200 ng	7–8	8–9
100 ng	8–9	9–10
50 ng	9–10	10–11
5 ng	12–13	13–14
1 ng	15–16	16–17
0.5 ng	16–17	17–18
0.25 ng	17–18	18–19
0.1 ng	19–20	20–21

9. Remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.
10. The yield and size distribution of the library should be checked on an Agilent Bioanalyzer before moving to the next step. If the yield is low compared to the positive control, please refer to the troubleshooting guide in the Appendix.

SAFE STOPPING POINT: At this stage, samples can be immediately processed for sequencing (proceed to Section 0) or stored frozen at –20°C for later processing.

B. Library Processing for Illumina Next-Generation Sequencing

1. Overview

This section contains guidelines for processing ThruPLEX DNA-Seq FLEX EF libraries for Illumina NGS. In some cases, recommended protocols are listed (e.g., library purification by AMPure XP beads or NucleoMag NGS Clean-up and Size Select) while in others, general guidelines are given. For more information, contact technical support.

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

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

AMPure XP or NucleoMag NGS Clean-up and Size Select Suspension is the recommended method of library purification.

NOTE: Do not use QIAquick cleanup or other silica-based filters for purification as this will result in an incomplete removal of primers.

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

Required reagents (supplied by the user): AMPure XP beads or NucleoMag NGS Clean-up and Size Select, 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 room temperature.
- Always use freshly prepared 80% (v/v) ethanol for Step 3 and Step 4 below.
- Resuspend the AMPure XP reagent or NucleoMag NGS Clean-up and Size Select Suspension by gentle vortexing until no visible pellet is present at the bottom of the container. The color of the liquid should appear homogeneous. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

1. Add 100 µl of AMPure XP reagent or NucleoMag NGS Clean-up and Size Select Suspension to 100 µl of amplified library (from Section V.A.3, Step 9) in a 200 µl tube, ensuring a 1:1 (v/v) ratio.
2. Mix by pipette 10 times to achieve a homogeneous solution; incubate for 5 min at room temperature.
3. Pulse-spin the sample(s) on a bench top centrifuge and place the tube in a magnetic stand. Wait for at least 10 min or until the beads are completely bound to the side of the tube(s) and the solution is clear.
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. Add 200 µl of 80% (v/v) ethanol to the pellet and let stand for 30 sec.
6. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard the supernatant.
7. Repeat Steps 4–6, for a total of two ethanol washes.
8. Allow beads to air dry for no more than ~5 min—do not allow them to crack.
9. Elute the DNA by resuspending the beads with 50 µl of l x TE buffer, pH 8.0.
10. Pulse-spin the sample(s) using a low speed, bench top centrifuge.
11. 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.
12. While keeping the sample(s) in the magnetic stand and without disturbing the pellet, transfer 48 µl of the supernatant with a pipette into a new tube.

SAFE STOPPING POINT: 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:

- Real-time PCR with a library quantification kit for Illumina NGS libraries, such as the Library Quantification Kit (Takara Bio, Cat. No. 638324)
- Fluorescence detection-based methods such as Qubit Fluorometer (Thermo Fisher Scientific) or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies)

- A fragment analyzer, such as the Agilent Bioanalyzer.

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

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

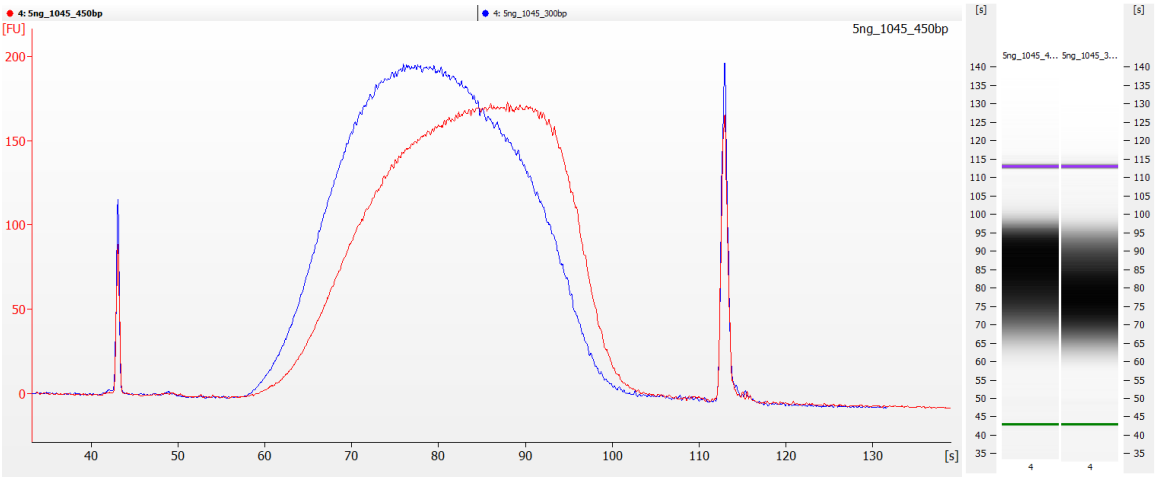

Figure 4. Bioanalyzer analysis of libraries prepared using ThruPLEX DNA-Seq FLEX EF. Libraries were prepared from 5 ng of Control Human gDNA using ThruPLEX DNA-Seq FLEX EF. Post library amplification, libraries were purified following the AMPure XP protocol (Section V.B.2). An aliquot of purified library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

Appendix. Troubleshooting Guide

Table 8. Troubleshooting guide for ThruPLEX DNA-Seq FLEX EF.

Problem	Potential cause	Suggested solutions
Low sample yield	No input DNA added	Quantify input before using the kit
	DNA mostly consists of single-stranded DNA (ssDNA); DNA contains impurities	Prior to library prep, evaluate DNA purity using $A_{260/230}$ and $A_{260/280}$ ratios; values outside the expected range (1.8–2.2 for $A_{260/230}$) may indicate contaminants that impact enzymatic reactions. If necessary, repurify DNA. Adhere to DNA sample requirements (Section IV.A)
	Insufficient library amplification	The remaining library can be further amplified to attain a higher yield (unless a plateau has been reached). Libraries which have not been purified can be further amplified with no extra reagents added after storage at 4°C for up to 6 hr or –20°C for up to 7 days. To perform additional amplification, spin down a tube or plate containing the library, transfer it to a thermal cycler, and perform an additional 2–3 cycles of the library amplification step of the PCR cycling program (Table 7) only.
NTC produces a yield similar to sample reaction products	NTC contaminated with DNA	Use a fresh control sample and check all reagents; replace kit if necessary. Clean area thoroughly and use PCR-dedicated plastics and pipettes.
After purification of the amplified library, Bioanalyzer traces show multiple peaks besides the markers	Input sample contains unevenly fragmented DNA of various sizes	If possible, quantify and check input DNA prior to using the kit. Sequencing is still recommended.
After purification of the amplified library, Bioanalyzer traces show broad peak(s) extending from 1,000 bp to greater than 10,000 bp, i.e., PCR bias.	Library was overamplified or the Bioanalyzer chip was overloaded. (This is common with high-sensitivity chips.)	Perform fewer PCR cycles during the Library Amplification Reaction. For high-sensitivity chips, load ~1–5 ng/µl. Repeat the Bioanalyzer run.
	Incorrect dilution of PEF1	Double-check PEF1 concentration used. Optimize dilution concentration with the Control Human gDNA and/or your control dsDNA sample.

(*table continued on next page*)

Problem	Potential cause	Suggested solutions
Following purification, average library size is <~400 bp	Overfragmentation has occurred, DNA template contains impurities such as salt or divalent ions.	If the DNA is isolated from cultured cells, culture medium must be completely removed by repeated wash steps.
	DNA could be nicked.	– Take care handling the sample during the procedure. Genomic DNA could be nicked physically during DNA purification or vortexing or pipetting – Do not overdry the DNA pellet during purification. Overdrying of the DNA pellet can also break the phosphodiester bond in DNA. – Make sure to keep the fragmentation enzyme mix and buffers on ice or prechilled cooling chamber and prepare mixes on ice. The fragmentation enzyme is active at room temperature
Average library size is >~500 bp	Underfragmentation has occurred	During template preparation (Section V.A.1), use less 1X PDF1 to dilute the PEF1 enzyme, i.e., shift left in the columns of Table 5 from the original experiment run when creating the Fragmentation Enzyme Dilution.

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