Takara Bio USA, Inc.

ThruPLEX® Plasma-Seq Kit User Manual

Cat. Nos. R400679, R400680, R400681, R400682
(112219)

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I. Introduction

Next-generation sequencing (NGS) of circulating cell-free DNA (cfDNA) from plasma is a rapidly growing application. Library preparation is a critical step in the NGS workflow (Figure 1) and has a direct impact on the quality of sequencing results. The process involves placing Illumina® sequencing adapters on DNA fragments and adding Illumina-compatible indexes for identification of individual samples. Multiple samples are then pooled (multiplexed) and sequenced in parallel.

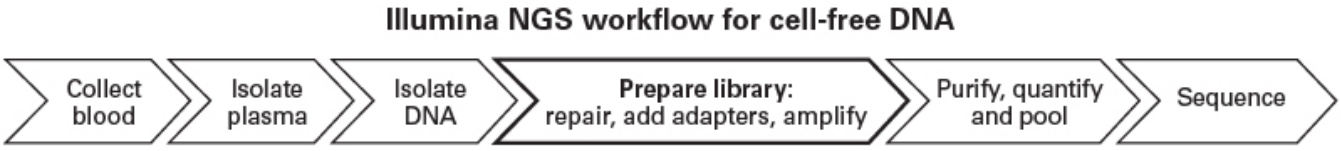

Figure 1. Illumina NGS workflow for cfDNA. Blood samples are collected, plasma is isolated, and cfDNA purified. ThruPLEX Plasma-Seq library preparation follows and consists of three steps: repair, ligation and amplification. Indexed libraries are purified, quantified, and pooled prior to sequencing on Illumina NGS platforms.

There are several key challenges in sequencing cfDNA from plasma. First, cfDNA is present in limited quantities in plasma samples. Second, the collected plasma samples can be highly variable in quality, DNA content, and composition. Lastly, the use of NGS samples necessitates careful tracking; a protocol in which the sample never leaves the tube is advantageous to ensure accurate sample tracking and to avoid contamination. The commitment to overcome these challenges is the core of the ThruPLEX Plasma-Seq Kit. It has been developed specifically for cfDNA derived from plasma by reformulating the repair and ligation reactions. The kit generates high-quality Illumina NGS libraries from variable amounts of cfDNA ranging from 1 to 30 ng with a standard protocol. The performance of ThruPLEX Plasma-Seq libraries is highly reproducible between replicates, sequencing runs, and from sample to sample. ThruPLEX Plasma-Seq's three-step, single-tube library preparation workflow (Figure 2) is the simplest in the industry, minimizing handling errors and loss of valuable samples.

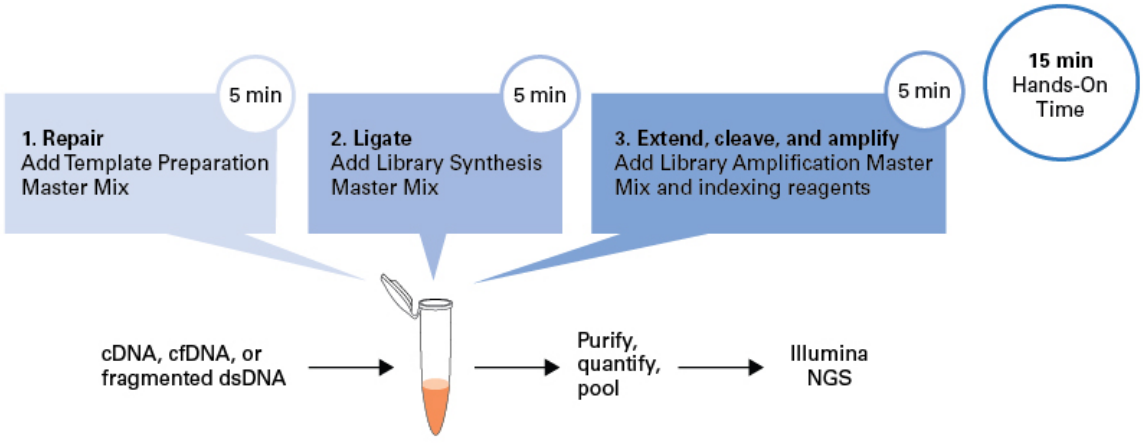

Figure 2. ThruPLEX Plasma-Seq single-tube library preparation workflow. The workflow consists of three simple steps that take place in the same PCR tube or well and eliminates the need to purify and transfer the sample material.

A. Overview

The ThruPLEX Plasma-Seq Kit is designed to generate DNA libraries for Illumina NGS from cfDNA isolated from plasma. The ThruPLEX Plasma-Seq chemistry is engineered and optimized to produce highly diverse libraries with reproducible sequencing performance from 1 to 30 ng of cfDNA. The entire three-step workflow takes place in a single tube or well in about 2 hours. No intermediate purification steps and no sample transfers are necessary, which prevent handling errors and loss of valuable samples.

The ThruPLEX Plasma-Seq Kit can be combined with multiple Takara Bio DNA indexing kits to generate NGS-ready libraries for multiplexing up to 384 samples. Once purified and quantified, the resulting libraries are ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols.

B. Principle

The ThruPLEX Plasma-Seq Kit is based on our patented ThruPLEX technology (Figure 3). 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, the cfDNA is repaired and yields molecules with blunt ends. In the next step, stem-loop adaptors with blocked 5' ends are ligated with high efficiency to the 5' end of the cfDNA, leaving a nick at the 3' end of the target fragment. The adaptors do not have single-strand overhangs and are prevented from ligating to each other, both of which contribute to nonspecific background found with many other NGS preparations. In the final step, the 3' ends of the cfDNA are extended to complete library synthesis and Illumina-compatible indexes are added through a high-fidelity amplification. Any remaining free adaptors are destroyed. Hands-on time and risk of contamination are minimized by using a single-tube protocol and eliminating intermediate purifications.

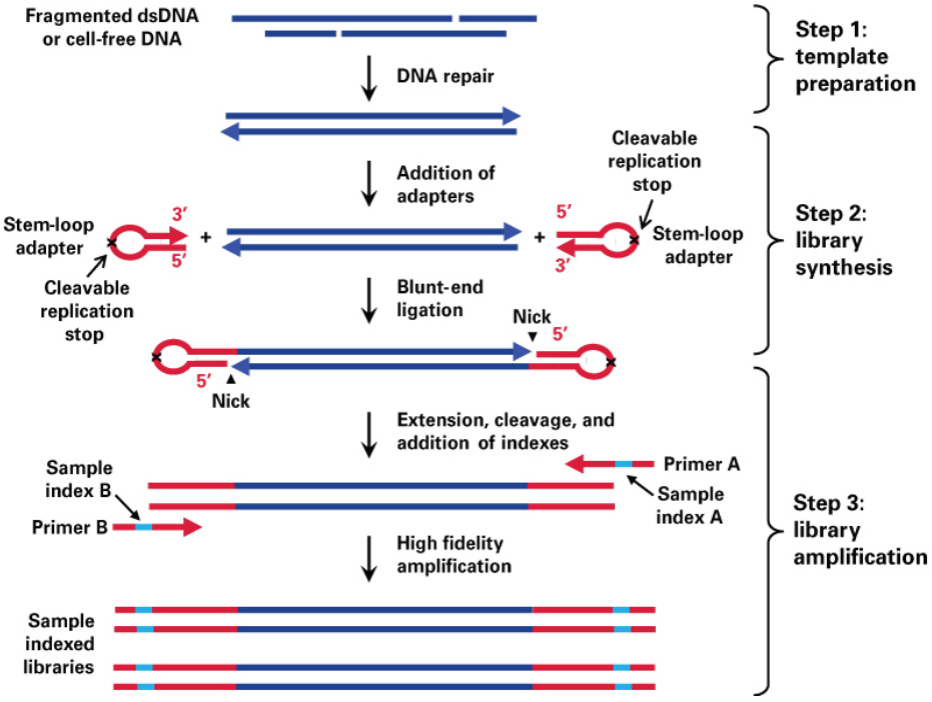

Figure 3. ThruPLEX Plasma-Seq technology. A three-step, single-tube reaction that starts with fragmented dsDNA or isolated cfDNA. Stem-loop adapters are blunt-end ligated to the repaired input DNA. These molecules are extended, then amplified using a high-fidelity polymerase to yield an indexed Illumina NGS library.

C. ThruPLEX Plasma-Seq Workflow

The highly streamlined ThruPLEX Plasma-Seq workflow (Figure 4) consists of the following three steps:

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

The three-step ThruPLEX Plasma-Seq workflow takes place in a single tube or well and is completed in about 2 hours.

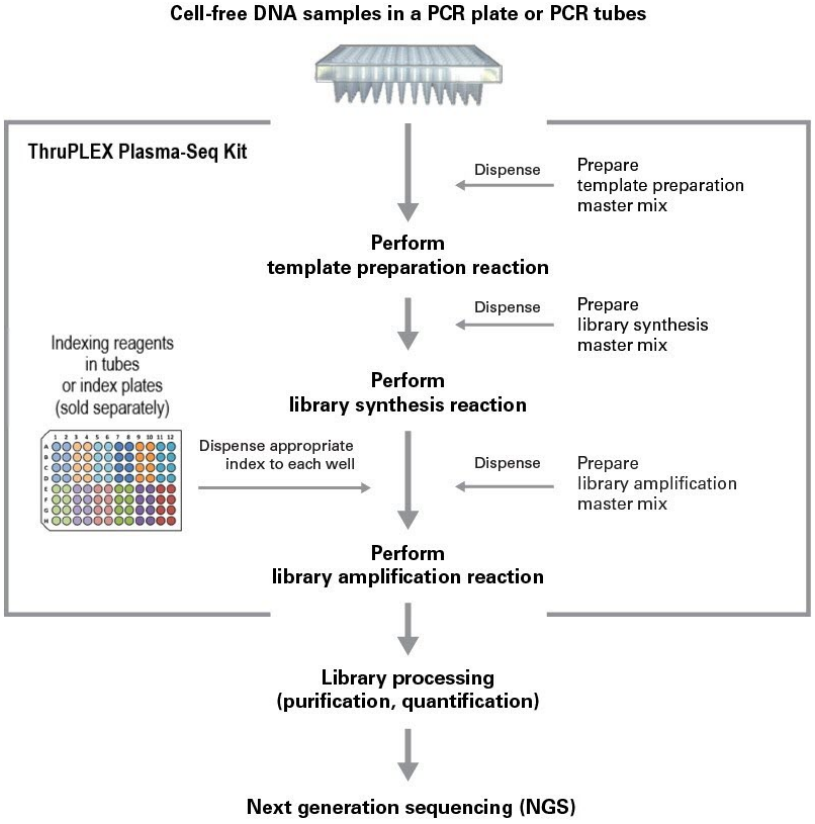

Figure 4. ThruPLEX Plasma-Seq library preparation workflow overview. Steps involved in ThruPLEX library preparation for Illumina NGS starting with isolated cfDNA.

II. List of Components

The ThruPLEX Plasma-Seq Kit contains sufficient reagents to prepare up to the specified number of reactions. Enough buffers and enzymes are provided to prepare master mixes for up to 4 separate uses. **Freeze-thaw of reagents should be limited to no more than four times**. Contents of the ThruPLEX Plasma-Seq Kit are not interchangeable with other Takara Bio products.

Table 1. ThruPLEX Plasma-Seq Kit Components

Name	Cap color	R400679 (24 rxns)	R400680 (48 rxns)	R400681 (96 rxns)	R400682 (480 rxns) (5 x R400681)
Template Preparation P Buffer	Red	105 µl	205 µl	405 µl	5 x 405 µl
Template Preparation P Enzyme	Red	25 µl	50 µl	105 µl	5 x 105 µl
Library Synthesis P Buffer	Yellow	65 µl	130 µl	255 µl	5 x 255 µl
Library Synthesis P Enzyme	Yellow	65 µl	130 µl	255 µl	5 x 255 µl
Library Amplification P Buffer	Green	545 µl	1,100 µl	2 x 1,100 µl	10 x 1,100 µl
Library Amplification Enzyme	Green	25 µl	50 µl	105 µl	5 x 105 µl
Nuclease-Free Water	Clear	500 µl	500 µl	500 µl	5 x 500 µl

IMPORTANT: The ThruPLEX Plasma-Seq Kit is shipped on dry ice and should be stored at –20ºC upon arrival.

III. General Considerations

A. Required Materials

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results.

- Barcoded primers (single or dual index)
  The ThruPLEX Plasma-Seq Kit is designed for high- or low-throughput applications and can be used with single-index or dual-index format barcoded primers for Illumina NGS. The following validated barcoded primers (sold separately)-can be used with ThruPLEX Plasma-Seq kits:

Table 2. Takara Bio DNA indexing kits

Takara Bio DNA single index kit (tubes)

Kit name	Number of reactions	Cat. No.
12S Set A	96 rxn	R400695
12S Set B	96 rxn	R400697

Takara Bio DNA unique dual index kit (tubes)

Kit name	Number of reactions	Cat. No.
24U Set A	48 rxn	R400665
24U Set B	48 rxn	R400666
24U Set C	48 rxn	R400667
24U Set D	48 rxn	R400668

Takara Bio DNA HT dual index kit

Kit name	Number of reactions	Cat. No.	Media type
24N	48 rxn	R400664	Tubes
96N Set A	96 rxn	R400660	Plate
96N Set B	96 rxn	R400661	Plate
96N Set C	96 rxn	R400662	Plate
96N Set D	96 rxn	R400663	Plate

Barcoded primers can also be used for low-level multiplexing of a small number of samples. It is important to select appropriate index combinations such that they are unique and meet Illumina-recommended compatibility requirements. Please refer to Illumina's technical manuals (Index Adapters Pooling Guide, Illumina Document # 1000000041074 v02, May 2018) for additional information.

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

  NOTE: See Thermal Cycler Considerations in Section III.E.

- Centrifuge
- PCR tubes or 96-well PCR plates and seals

  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 prevent cross-contamination and eliminate evaporation during cycling conditions. Evaporation could reduce the robustness and reproducibility of the reactions.

- 1.5 ml low adhesion microcentrifuge tube, natural (USA Scientific, Cat. No. 1415-2600)
- 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
- 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.

B. Optional Materials

The following reagents are not required but recommended.

- EvaGreen fluorescent dye, 20X in water (Biotium, Cat. No. 31000-T)
- Fluorescein Calibration Dye (Bio-Rad Laboratories, Cat. No. 170-8780)
- Reference Dye (if necessary by real time instrument)
- qPCR-based library quantification kit for Illumina NGS libraries

C. Starting Material

Table 3. DNA Sample Requirements

DNA Sample Requirements	
Source	Plasma
Type	Isolated cfDNA
Recommended Input Amount	1 ng to 30 ng
Input Volume	10 µl
Input Buffer	≤10 mM Tris, ≤0.1 mM EDTA

Blood collection and plasma preparation
Blood collection and plasma preparation protocols for ThruPLEX Plasma-Seq can be accessed through the learning center at takarabio.com.

Sample type
The ThruPLEX Plasma-Seq Kit is optimized for cell-free DNA isolated from human plasma. This kit is **not** for use with single-stranded DNA (ssDNA) or RNA.

cfDNA isolation
The ThruPLEX Plasma-Seq Kit was optimized with cell-free DNA.

Input DNA amount
The recommended input amount is 1 ng to 30 ng of isolated cfDNA quantified by Qubit Fluorometer or equivalent methods. Libraries have been generated from input amounts less than 1 ng and over 30 ng, requiring the number of PCR cycles to be optimized. Contact technical support at technical_support@takarabio.com for additional information.

Input volume
The maximum input sample volume is 10 µl. If a sample is in a larger volume, the DNA must be concentrated to 10 µl or less. Care should be taken to ensure the buffer concentration is appropriate (see below).

Input buffer
The concentrations of Tris and EDTA in the buffer containing the input DNA must not exceed 10 mM and 0.1 mM, respectively. Avoid phosphate-containing buffers.

Fragment size
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 the Agilent Bioanalyzer.

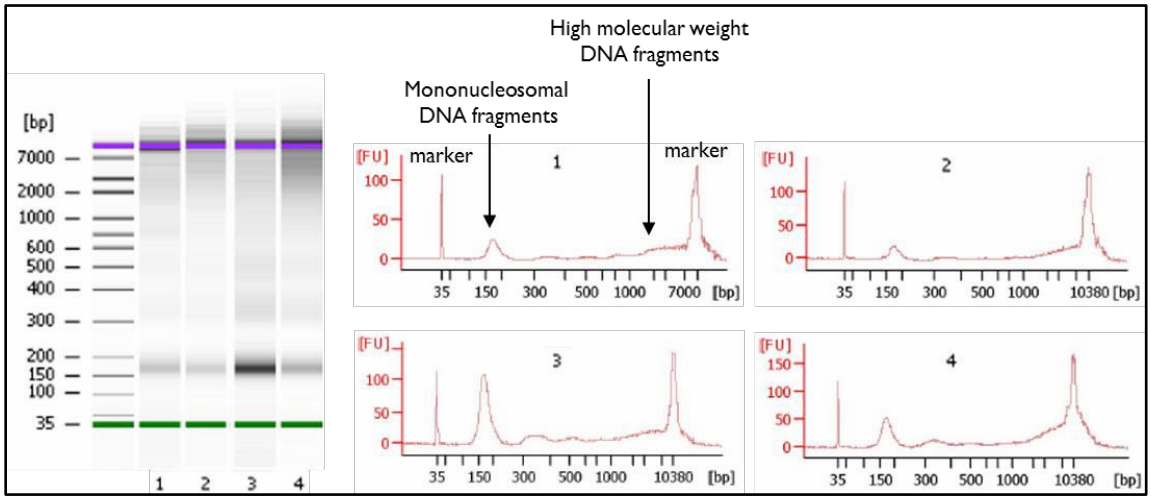

Figure 5. Fragment size distribution of cfDNA isolated from Plasma. cfDNA from four different human plasma samples was isolated and analyzed using the Agilent Bioanalyzer. One distinct feature of the isolated cfDNA is a peak (or band) centered around 170 bp and a distribution of higher molecular weight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

D. 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.

E. Thermal Cycler Considerations

Thermal cycling and heated lid

Use a thermal cycler equipped with a heated lid that can handle 50-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.B) to the reaction. 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 IV.B.2**). 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 IV.A.3**). For some real-time instruments, calibration dye may not be needed; please refer to the real-time thermal cycler instrument's manual.

F. Positive and Negative Controls

Include appropriate positive and negative controls in the experimental design to verify that reactions proceed as expected.

Always prepare fresh dilutions of reference DNA. Include negative controls (No Template Control, NTC) in low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) or nuclease-free water.

The positive control and experimental samples should produce indexed libraries, while the NTC should not express significant amplification.

G. 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 ~5% excess of each master mix to allow for pipetting losses. Each kit contains sufficient reagents to prepare master mixes up to four separate times.

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, vortex briefly and centrifuge prior to use.
3. Keep all the components and master mixes on ice.
4. Once the master mix is prepared, thoroughly mix the contents several times with a pipette while avoiding introduction of air bubbles and 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 minutes of the previous step's cycling protocol and kept on ice until use.

H. Using Illumina Experiment Manager

Make sure the latest version of the Illumina Experiment Manager (IEM) is installed (version 1.15 or later). Prior to starting the ThruPLEX Plasma-Seq Library Preparation Protocol (**Section IV. A**), create a Sample Sheet in the IEM to select and validate appropriate indexes to use in your experiments. Refer to the Index Adapters Pooling Guide for additional information (Illumina, Document No. 100000004107 v02, 2018).

I. Target Enrichment

ThruPLEX Plasma-Seq is compatible with the major exome and target enrichment products, including Agilent SureSelectXT, XT2 and QXT, Roche NimbleGen SeqCap EZ, IDT xGEN lockdown probe and Illumina Nextera® Rapid Capture Exome Enrichment Kit. ThruPLEX Plasma-Seq target enrichment protocols can be accessed through the learning center at takarabio.com.

IV. Protocols

A. ThruPLEX Plasma-Seq Library Preparation Protocol

1. Template Preparation Step

Template Preparation Reagents

Reagent	Cap Color
Template Preparation P Buffer	Red
Template Preparation P Enzyme	Red

NOTE: Assemble all reactions in thin-wall 96-well PCR plates or PCR tube(s) that are compatible with the thermal cycler or real-time thermal cycler used.

1. Prepare samples as described below.
   - **Samples:** Dispense 10 µl of isolated cfDNA into each PCR tube or well of a PCR plate.
   - **Positive control reactions using reference DNA:** If desired, include a positive control DNA.
   - **Negative control reactions (No Template Controls; NTCs):** If desired, assemble NTCs with 10 µl of nuclease-free water or low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

   NOTE: The maximum volume of DNA cannot exceed 10 µl.

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

   4 µl Template Preparation P Buffer
   1 µl Template Preparation P Enzyme
   5 µl Total Volume

   NOTE: Prepare 5% excess to allow for pipetting losses.

3. Assemble the **Template Preparation Reactions Mixture** as shown in the table below. To each 10 µl sample from Step 1 above, add 5 µl of the **Template Preparation Master Mix.**

   10 µl cfDNA Sample
   5 µl Template Preparation P Master Mix
   15 µl Total Volume

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 a heated lid set to 101°C–105°C. Perform the **Template Preparation Reaction** using the conditions in the table below:

Template Preparation Reaction	
Temperature	Time
22°C	25 min
55°C	20 min
22°C	Hold for ≤2 hr

8. After the thermal cycler reaches 22°C, remove the plate or tube(s) and centrifuge briefly.
9. Proceed to the Library Synthesis Step.

NOTE: Following the Template Preparation Step, continue to Library Synthesis Step in the same plate or tube(s).

2. Library Synthesis Step

Library Synthesis Reagents

Reagent	Cap Color
Library Synthesis P Buffer	Yellow
Library Synthesis P Enzyme	Yellow

Library Synthesis Protocol

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

2.5 µl Library Synthesis P Buffer
2.5 µl Library Synthesis P Enzyme
5 µl Total Volume

NOTE: Prepare 5% excess to allow for pipetting losses.

2. Remove 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 5 µl of the **Library Synthesis P Master Mix**.

15 µl Template Preparation reaction product
5 µl Library Synthesis P Master Mix
20 µl Total Volume

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 a heated lid set to 101°C–105°C. Perform the **Library Synthesis Reaction** using the cycling conditions in the table below:

Library Synthesis Reaction	
Temperature	Time
30°C	40 min
4°C	Hold for ≤30 min

8. After the thermal cycler reaches 4°C remove the plate or tube(s) and centrifuge briefly.
9. Continue to the **Library Amplification Step**.

NOTE: Following the Library Synthesis Step, continue Library Amplification Reaction in the same plate or tube(s) maintained at 4°C.

3. Library Amplification Step

Multiple stages occur during the **Library Amplification Reaction** (see Table 4 in Step 8 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 steps to be completed correctly, with no denaturation step until Stage 3.**

Illumina-compatible indexes are incorporated into the template library in Stage 4 using four amplification cycles. In Stage 5, the resulting template is amplified; the number of cycles required at this stage is dependent on the amount of cell-free DNA used. Samples are cooled to 4°C in Stage 6, after which they are pooled and purified or stored at −20°C.

NOTE: Indexed primers sold separately.

Selection of the optimal number of cycles for library amplification (▲Stage 5):

The number of PCR cycles required at Stage 5 of the Library Amplification Reaction is dependent on the amount of cfDNA used. Use the table below as a guide for selecting the number of PCR cycles.

▲ Stage 5 Amplification Guide	
Input DNA	**Number of cycles required to generate 500–1,000 ng library**
30 ng	7–8
5 ng	9–10
1 ng	13–14

- **Yield:** The amount of amplified library can vary depending upon sample condition and composition. When starting with Qubit-quantified cfDNA and following the recommended number of amplification cycles, the typical yields range from 500 ng to 1000 ng.

NOTE: Libraries have been generated from input amounts from less than 1 ng up to 100 ng gDNA, requiring the number of PCR cycles to be optimized. Contact technical support for further information (technical_support@takarabio.com).

Library Amplification Reagents

Reagent	Cap Color
Library Amplification P Buffer	Green
Library Amplification Enzyme	Green
Nuclease-Free Water	Clear
Fluorescent Dyes (optional)	
Indexing Reagents (sold separately)	

Library Amplification Protocol

1. Prepare Indexing Reagents
   - Remove Indexing Reagents from freezer and thaw for ten minutes on the bench.
   - Mix and spin Indexing Reagents briefly in a tabletop centrifuge to collect contents to the bottom.
2. Prepare the **Library Amplification P Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used:

21.5 µl Library Amplification P Buffer
1 µl Library Amplification Enzyme
2.5 µl Nuclease – Free Water (or fluorescent dye mix *)
25.0 µl Total Volume

NOTES:

- Prepare 5% excess to allow for pipetting losses.
- **If monitoring in real time:** Fluorescent 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 2.5 µl.

  Example:

  Mix 90 µl of 20X EvaGreen dye (Biotium, Cat. No. 31000-T, EvaGreen Dye, 20X in water) with 10 µl of 1:500 dilution of Fluorescein (Bio-Rad Laboratories, Cat. No. 170-8780, Fluorescein Calibration Dye). Add 2.5 µl of this mix per reaction to prepare the Library Amplification Master Mix.
- **If not monitoring in real time:** There is no need to add the dyes; use 2.5 µl of nuclease-free water per reaction in the Library Amplification Master Mix.

3. Remove the seal on the PCR plate or open the tube(s) containing the Library Synthesis Reaction Product.
4. Add 25 µl of the **Library Amplification P Master Mix** to each well or tube.

5. Add 5 µl of the appropriate Indexing Reagent to each well or tube:

20 µl Library Synthesis Reaction Product
25 µl Library Amplification P Master Mix
5 µl Indexing Reagent
50 µl Total Volume

If using an Index Plate:

- 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.
- Use a clean pipette tip to pierce the seal above the specific Indexing Reagent on the Index Plate; discard the tip used for piercing.
- Use a clean pipette tip to collect 5 µl of the Indexing Reagent and add to the reaction mixture.

6. Mix thoroughly with a pipette. Avoid introducing 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.

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

8. Return the plate or tube(s) to the thermal cycler with a heated lid set to 101°C–105°C. Perform **Library Amplification Reaction** using the cycling conditions in the tables below.

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

Table 4. Thermal Cycling Conditions for the Library Amplification Reaction

Library Amplification Reaction				
	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
Addition of Indexes	4	98°C	20 sec	4
		67°C	20 sec	
		72°C	40 sec	
Library Amplification	5	98°C	20 sec	7–14 (see ▲ Stage 5 Amplification Guide)
		72°C*	50 sec	
	6	4°C	Hold	1

***Acquire fluorescence data at this step, if monitoring amplification in real-time.**

▲ Stage 5 Amplification Guide	
DNA Input (ng)	**Number of cycles required to generate 500–1000 ng library**
30	7–8
5	9–10
1	13–14

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

NOTE: It is highly recommended to check the yield of DNA library before the Bead purification step on the Agilent Bioanalyzer. If DNA yield is less than expected, extra PCR cycles can be added. During this time, samples can be stored at 4 degrees for about 6 hrs.

At this stage, samples can be processed for next-generation sequencing (NGS) immediately or stored frozen at −20°C for up to 2 weeks. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to **Section IV.B**.

B. Library Processing for Illumina Next-Generation Sequencing

1. Overview

This section contains guidelines for processing ThruPLEX Plasma-Seq libraries for Illumina NGS. In some cases, recommended protocols are listed (Library Purification by AMPure XP Beads) while in others, general guidelines and manufacturer's instructions are referred. For more information, contact technical support at technical_support@takarabio.com.

Libraries prepared from each sample will contain the specific indexes selected at the time of amplification. Follow the recommended workflow (solid arrows) in Figure 6 to process the libraries for Illumina NGS. Alternative workflow paths (dashed arrows) may be followed as needed. If libraries are prepared from similar samples with equivalent input amounts, the replicates can be pooled into one tube for further processing. This "pooled" library is then purified using AMPure XP to remove unincorporated primers and other reagents. 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 pooling, purification, quantification, and sequencing are described in the following sections.

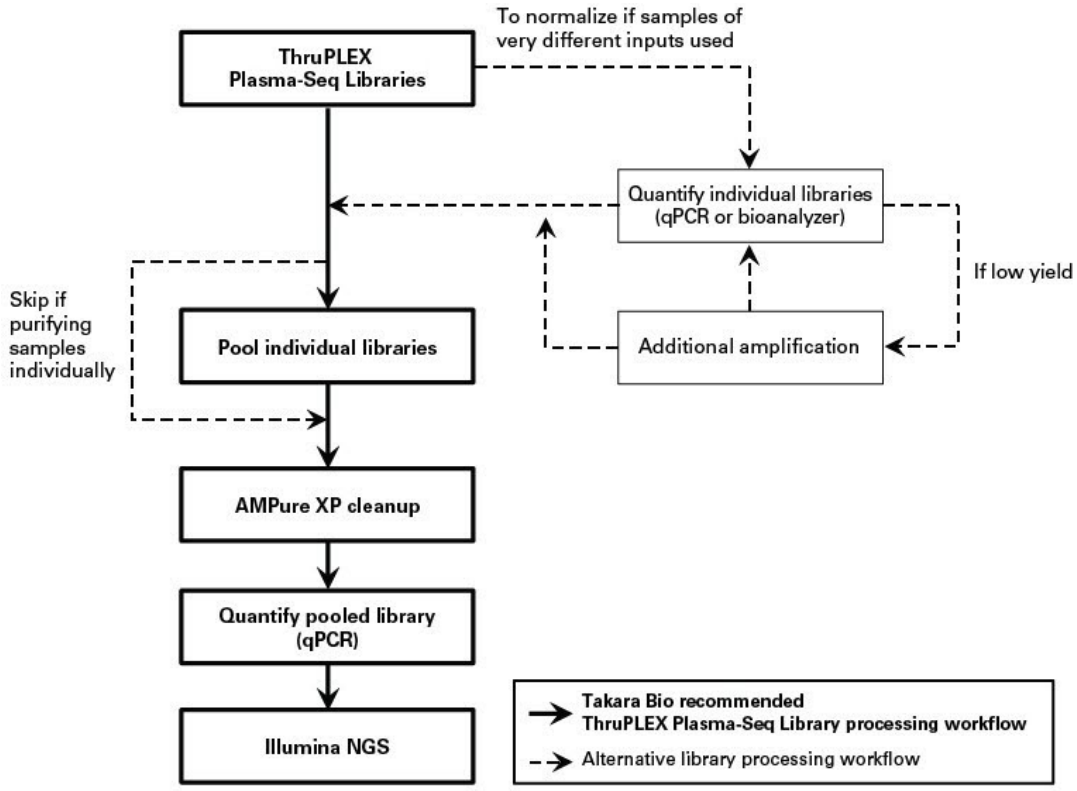

Figure 6. Workflow for processing the ThruPLEX Plasma-Seq amplified libraries for Illumina NGS.

2. Library Quantification

There are several approaches available for library quantification including real-time PCR, UV absorption,

fluorescence detection, and sizing and quantification using the Agilent Bioanalyzer. It is important to understand the benefits and limitations of each approach. Real-time PCR-based approaches quantify the library molecules that carry the Illumina adapter sequences on both ends and, therefore, reflect the quantity of the clustering competent library molecules. This approach assumes a relatively uniform size distribution of input DNA used for library construction.

UV absorption/fluorescence detection-based methods (i.e., Nanodrop (Thermo Scientific), Qubit Fluorometer (Thermo Fisher), or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies)) quantify total double-stranded nucleic acid concentration. These methods do not discriminate adapter presence and offer no information about the size of the library molecules. The Agilent Bioanalyzer system provides sizing and quantitation information about the library analyzed, but not about the clustering competency.

Quantify ThruPLEX Plasma-Seq libraries by real-time qPCR

Use the appropriate instrument-specific library quantification kit for Illumina NGS libraries. Dilute 2–5 µl of the library 100,000-fold and use this as the template for the quantification procedure. Since the adapters result in about 140 bp increase in the DNA fragment size, adjust the length accordingly to calculate the concentration of your library. For example, for a typical cfDNA input with fragments of about 170 bp in size, use 310 bp as the approximate size for calculating library concentration.

NOTE: No purification of the template is necessary prior to qPCR due to the large dilution factor.

Quantify ThruPLEX Plasma-Seq libraries using the bioanalyzer

Dilute an aliquot of each library in TE buffer to 100 pg/µl to 10 ng/µl. Load a 1-µl aliquot of this diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, Cat. No. 5067-4626).

3. Additional Amplification

If the results show less than desirable yield, the remaining library can be further amplified to attain a higher yield. The additional amplification can only be performed on **unpurified** libraries. ThruPLEX Plasma-Seq libraries can be further amplified with no extra reagents added after storage at 4°C for up to 6 hours or –20°C for up to 7 days. To perform this additional amplification, spin down the tube or plate containing the library (after thawing if the plate or tube was stored at –20°C), transfer it to a thermal cycler, and perform 2–3 PCR cycles as follows:

Number of Cycles	Temperature	Time
2–3 cycles	98°C	20 sec
	72°C	50 sec
1 cycle	4°C	Hold

4. Library Pooling for Purification

When pooling libraries for sequencing, use the Illumina Experiment Manager (IEM) or BaseSpace to record information about your samples **before** beginning the ThruPLEX Plasma-Seq Library Preparation Protocol.

Individual ThruPLEX Plasma-Seq libraries containing different indexes can be pooled at desired molar ratios to allow multiplex sequencing. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of the individual libraries, each containing a unique index.

Typically, a 10-µl aliquot from each library is adequate and the remainder of the library can be stored at −20°C. The total volume obtained at the end of pooling will vary depending on the number of libraries pooled. For example, if 12 libraries are pooled, then the final volume of the pool is 120 µl; if 48 libraries are pooled, then the volume is 480 µl. A 100-µl aliquot of this pooled library is sufficient for AMPure XP purification purposes.

Some Illumina sequencing systems require balanced index combinations to ensure proper image registration and accurate demultiplexing of the pooled samples. Please see Illumina Index Adapters Pooling Guide (1000000041074 v02) for guidelines on selecting the appropriate indexes for pooling and multiplexing.

5. 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 is application dependent and determines the size-selection characteristics of the library. 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.

Library purification reagents (supplied by the user)

Reagent
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

AMPure XP protocol

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 4 (below).
- Resuspend the AMPure XP reagent by gentle vortex until no visible pellet is present at the bottom of the container.

1. In a 1.5-ml tube, combine AMPure XP reagent with an aliquot of the pooled library at 1:1 (v/v) ratio.
2. Mix by pipetting 10 times to achieve a homogeneous solution; incubate for 5 min at room temperature.
3. Place the tube on a magnetic stand for 2 min or until the beads are completely bound to the side of the tube(s), and the solution is clear.
4. With the tube in the magnetic stand:
   a. Without disturbing the pellet, use a pipette to remove and discard the supernatant.
   b. Add 200 µl of 80% (v/v) ethanol to the pellet; incubate for 30 sec.

      NOTE: The volume of 80% (v/v) ethanol should be at least the volume of the sample plus AMPure XP reagent (total volume from Step 1).

   c. Without disturbing the pellet, use a pipette to remove and discard the supernatant.
5. Repeat Step 4.
6. Incubate the tube for 2–5 min at room temperature with the cap open to evaporate residual ethanol. **DO NOT OVER DRY THE PELLET(S)**.
7. Remove the tube from the magnetic stand.
8. Resuspend the beads in an appropriate volume of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) or nuclease-free water.

   NOTE: Resuspend in nuclease-free water if a vacuum concentrator will be used to concentrate the sample.

9. Mix by pipetting 10 times to achieve a homogeneous solution; incubate for 2 min at room temperature.
10. Place the tube on a magnetic stand for 1–2 min or until the beads are captured and the solution is clear.
11. With the tube in the magnetic stand and without disturbing the pellet, transfer the supernatant with a pipette into a new tube.

    NOTE: If not used immediately, the purified library can be stored at −20 °C.

6. Sequencing Recommendations

The ThruPLEX Plasma-Seq Kit generates libraries that are ready for cluster amplification and sequencing on Illumina NGS platforms using standard Illumina reagents and protocols for multiplexed libraries. Libraries prepared using the ThruPLEX Plasma-Seq Kit result in a size distribution of library fragments centered at about 310 bp (Figure 7) when analyzed using the Agilent Bioanalyzer.

To achieve optimal cluster density on the Illumina flow cell, it is important to adjust the DNA concentration used for clustering based on these preferences. For example, if using NextSeq®, load 1.8 pM ThruPLEX Plasma-Seq libraries.

Illumina recommends adding 1% PhiX control for most libraries. PhiX is a small genome that provides a balanced and diverse library to prevent sequencing problem and that can be quickly aligned to calculate error rates. For low diversity libraries and if experiencing sequencing issues, increase the PhiX control spike-in as recommended in Illumina's bulletin: "How much PhiX spike-in is recommended when sequencing low diversity libraries on Illumina platforms?" (04/07/2017).

For sequencing on the HiSeq®, please refer to Illumina's technical note Using a PhiX Control for HiSeq Sequencing Runs (Illumina, Pub. No. 770-2011-041). For sequencing on the MiSeq®, instructions for preparing a PhiX control can be found in Illumina's guide on Preparing Libraries for Sequencing on the MiSeq (Illumina, Part No. 15039740 v06, 2018).

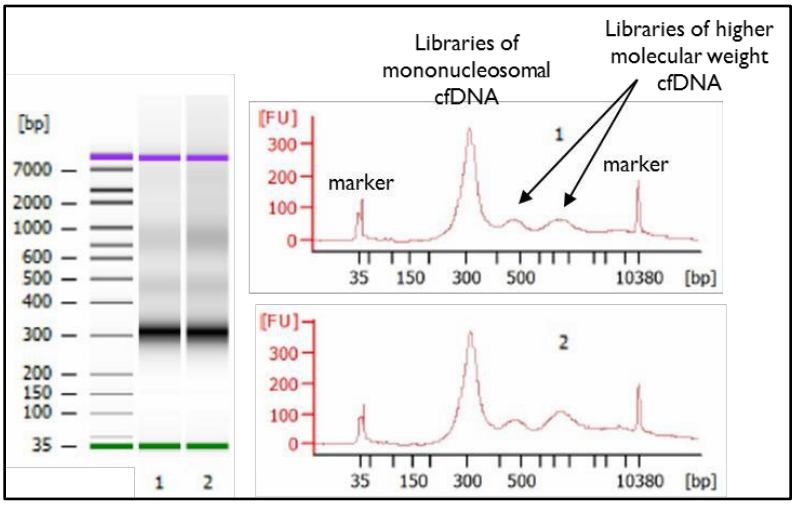

Figure 7. Bioanalyzer analysis of libraries prepared using ThruPLEX Plasma-Seq. cfDNA from plasma was isolated using the QIAamp Circulating Nucleic Acid Kit. Libraries were prepared for two independent samples using the ThruPLEX Plasma-Seq Kit and analyzed using the Agilent Bioanalyzer after library amplification. One distinct feature of the amplified libraries is a dominant peak (or intense band) around 310 bp containing library products of the mononucleosomal DNA fragments and a distribution of higher molecular weight library products.

Appendix A. Troubleshooting Guide

Table 5. Troubleshooting Guide

Problem	Potential Cause	Suggested Solutions
Sample amplification curve looks like No Template Control (NTC) amplification curve or does not produce amplified product	No input DNA added	Quantitate input before using the kit
	Incorrect library template used (e.g., RNA, ssDNA)	Adhere to DNA Sample Requirements (Section III.C.)
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 Clean area thoroughly and use PCR-dedicated plastics and pipettes
After purification of the amplified library, Bioanalyzer traces show broad peak(s) extending from less than 1,000 bp to greater than 1,000 bp	Library over-amplified or Bioanalyzer chip overloaded (common for high-sensitivity chips)	Perform fewer PCR cycles at Stage 5 of the Library Amplification Reaction For high sensitivity chips, load 100 pg/µl to 10 ng/µl. Repeat Bioanalyzer run
Low yield	DNA mostly consists of single-stranded DNA (ssDNA)	ssDNA is irreparable thus not suitable for library construction
	DNA concentration wasn't determined accurately	DNA yield can be assessed using various methods including absorbance (NanoDrop), agarose gel electrophoresis, use of fluorescent DNA-binding dyes (Qubit), capillary electrophoresis (Agilent Bioanalyzer).
	DNA contains impurities	Impurities in DNA can lead to inaccurate measurement of DNA concentration and could potentially inhibit subsequent reactions
	DNA is not fragmented	DNA larger than 1 kb must be fragmented. The acceptable size for library construction ranges between 50 bp to 1000 bp

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