Takara Bio USA

ThruPLEX® DNA-Seq Kit User Manual

Cat. Nos. R400674, R400675, R400676 & R400677
(082124)

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I. Introduction

A. Overview

The **ThruPLEX DNA-Seq Kit** (R400674, R400675, R400676 & R400677) is designed to provide up to 384 indexed libraries for higher multiplexing capabilities on Illumina® NGS platforms. ThruPLEX DNA-Seq chemistry is engineered and optimized to generate DNA libraries with high molecular complexity from the lowest input amounts. Only 50 pg to 50 ng of fragmented double-stranded DNA is required for library preparation. 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 to prevent handling errors and loss of valuable samples. Providing high library diversity, ThruPLEX DNA-Seq libraries excel in target enrichment performance and deliver high-quality sequencing results.

The ThruPLEX DNA-Seq Kit can be combined with multiple DNA Indexing Kits to generate NGS-ready libraries for multiplexing up to 384 samples. Once purified and quantified, the resulting library is 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 in ChIP-seq and with small fragments of DNA such as damaged DNA from formalin-fixed paraffin-embedded (FFPE) tissue.

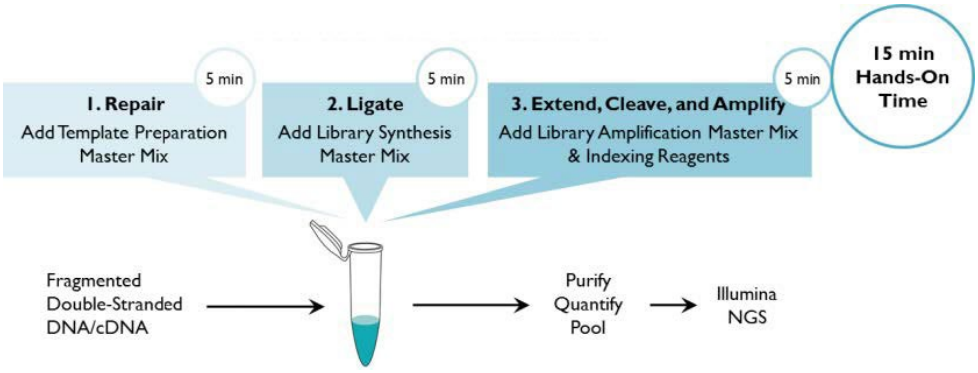

Figure 1. ThruPLEX DNA-Seq single-tube library preparation workflow. The ThruPLEX DNA-Seq workflow consists of three simple steps that take place in the same PCR tube or well, eliminating the need to purify and transfer the sample material.

B. Principle

The ThruPLEX DNA-Seq Kit is based on our patented ThruPLEX technology (Figure 2). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, ThruPLEX uses stem-loop adapters to construct high-quality libraries in a fast and efficient workflow. In the first step, Template Preparation, the DNA 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 genomic DNA, leaving a nick at the 3' end. The adaptors cannot ligate to each other and do not have single-strand tails, both of which contribute to non-specific background found with many other NGS preparations. In the final step, the 3' ends of the genomic DNA are extended to complete library synthesis, and Illumina-compatible indexes are added through a high-fidelity amplification. Any remaining free adaptors are destroyed. Hands-on time and the risk of contamination are minimized by using a single tube and eliminating intermediate purifications.

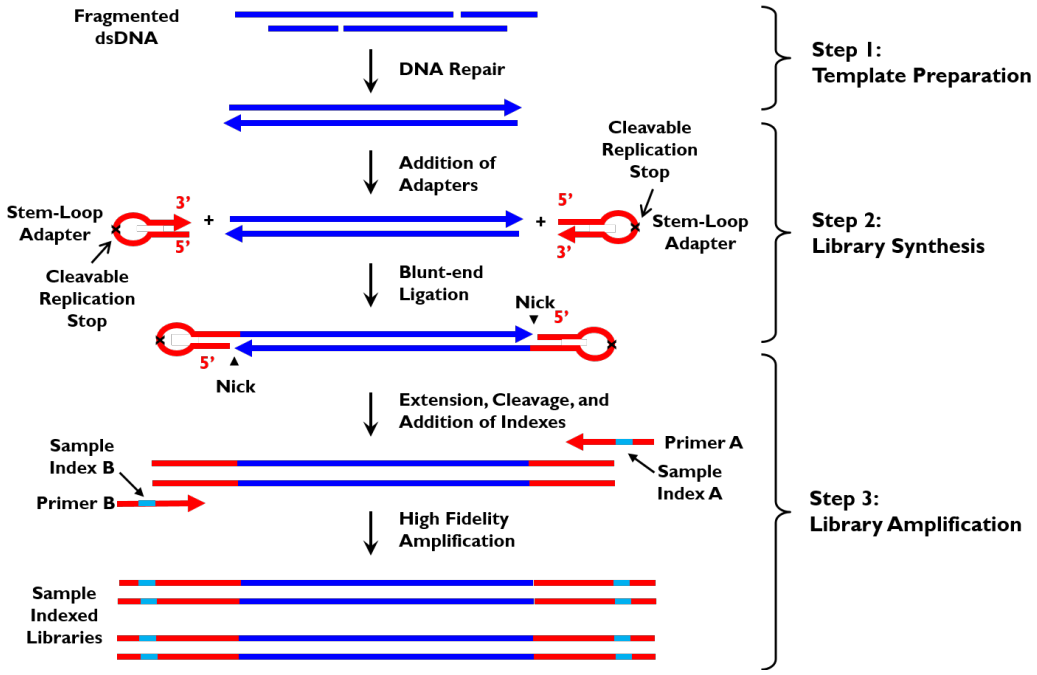

Figure 2. ThruPLEX DNA-Seq technology. A three-step, single-tube reaction starts with fragmented double-stranded DNA (0.05 ng to 50 ng). Stem-loop adapters are blunt-end ligated to repaired input DNA. These molecules are extended and then amplified to include barcodes using a high-fidelity polymerase, yielding an indexed Illumina NGS library.

C. ThruPLEX DNA-Seq Workflow

The ThruPLEX DNA-Seq Kit 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 adaptors, 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 workflow takes place in a single tube or well and is completed in about 2 hours.

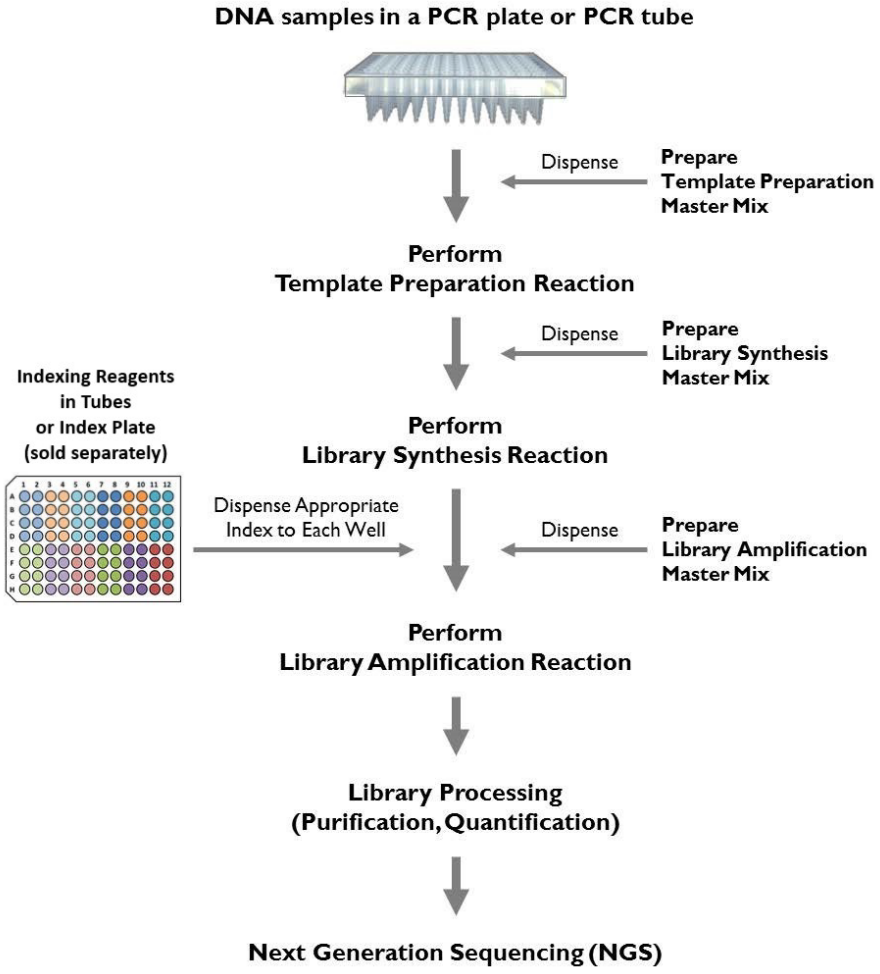

Figure 3. ThruPLEX DNA-Seq library preparation workflow overview. Steps involved in ThruPLEX library preparation for Illumina NGS starting with fragmented DNA.

II. List of Components

ThruPLEX DNA-Seq Kit (R400674, R400675, R400676 & R400677) consists of the following components. These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results. Please make sure to spin down tubes to collect all the liquid at the bottom before first use.

Table 1. ThruPLEX DNA-Seq Kit components.

ThruPLEX DNA-Seq Kit (Store at –20°C)	Cap color	R400674 (24 rxns)	R400675 (48 rxns)	R400676 (96 rxns)	R400677 (480 rxns) (5X R400676)
Template Preparation D Buffer	Red	50 µl	105 µl	205 µl	5 x 205 µl
Template Preparation D Enzyme	Red	25 µl	50 µl	105 µl	5 x 105 µl
Library Synthesis D Buffer	Yellow	25 µl	50 µl	105 µl	5 x 105 µl
Library Synthesis D Enzyme	Yellow	25 µl	50 µl	105 µl	5 x 105 µl
Library Amplification D Buffer	Green	630 µl	1,260 µl	2 x 1,275 µl	10 x 1,275 µl
Library Amplification Enzyme	Green	25 µl	50 µl	105 µl	5 x 105 µl
Nuclease-Free Water	Clear	500 µl	500 µl	1,000 µl	5 x 1,000 µl

III. Additional Materials Required (Not Provided)

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

Table 2. Unique Dual Index Kit (plates; 12.5 µM total/oligo pair)

Index set	Number of reactions	Cat. No.
1–96	96 rxns	634752
97–192	96 rxns	634753
193–288	96 rxns	634754
289–384	96 rxns	634755
1–24	24 rxns	634756

Table 3. DNA Single Index Kit (tubes; 5 µM total/oligo pair)

Index set	Number of reactions	Cat. No.
12S Set A	96 rxns	R400695
12S Set B	96 rxns	R400697

Table 4. DNA Unique Dual Index Kit (tubes; 5 µM total/oligo pair)

Index set	Number of reactions	Cat. No.
24U Set A	48 rxns	R400665
24U Set B	48 rxns	R400666
24U Set C	48 rxns	R400667
24U Set D	48 rxns	R400668

Barcoded primers can also be used for low-level multiplexing of a small number of samples. It is important to select unique index combinations that meet Illumina-recommended compatibility requirements. Please refer to Illumina's technical manuals (Index Adapters Pooling Guide, Illumina Document # 1000000041074-v8) for additional information.

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

  NOTE: See "Thermal Cycler Considerations", Section IV.C.

- Centrifuge
- 96-well nuclease-free thin-wall PCR plates or PCR tubes

  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 can reduce 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]
- 80% (v/v) ethanol: freshly made for each experiment
- Magnetic Separator
- 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

The following reagents are not required but recommended:

- Single-donor reference DNA (positive control)
- EvaGreen fluorescent dye, 20X in water (Biotium, Cat. No. 31000-T)
- 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: Library Quantification Kit (Takara Bio, Cat. No. 638324)

IV. General Considerations

A. Starting Material

Table 5. DNA sample requirements.

DNA Sample Requirements	
Nucleic Acid	Fragmented double-stranded DNA or cDNA
Source	Cells, plasma, urine, other biofluids, FFPE, tissues, fresh tissues, frozen tissues
Type	Mechanically sheared; enzymatically fragmented; ChIP DNA; low-molecular-weight cell-free DNA
Molecular Weight	<1,000 bp
Input Amount	50 pg to 50 ng
Input Volume	10 µl
Input Buffer	≤10 mM Tris, ≤0.1 mM EDTA

DNA format

Fragmented double-stranded DNA (gDNA or cDNA), chromatin immunoprecipitates (ChIP), degraded DNA from sources such as FFPE, plasma, or other biofluids are suitable. This kit is not for use with single-stranded DNA (ssDNA) or RNA.

Input DNA amount
Input DNA in the range of 50 pg to 50 ng can be used as starting material. For deep Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) using human gDNA, FFPE, or plasma DNA, greater than 10 ng of input DNA is recommended to achieve a highly diverse library. For sequencing samples with reduced complexity, such as cDNA, ChIP DNA, bacterial DNA, or targeted genomic regions, lower input amounts (picogram levels) can be used.

Fragment size
The optimal DNA fragment size is less than 1,000 bp. The ThruPLEX DNA-Seq Kit is a ligation-based technology and adapters added during the process result in an approximately 140 bp increase in the size of each DNA template fragment. Library molecules with shorter inserts (200–300 bp) tend to cluster and amplify more efficiently on the Illumina flow cell. Depending on the application and requirements, the AMPure purification step following the final step (Library Amplification) can be replaced with a size-selection step to remove unwanted fragments.

Input volume
The maximum input sample volume is 10 µl. If a sample is in a larger volume, the DNA must be concentrated into 10 µl or less. Alternatively, the sample may be split into 10 µl aliquots; processed in separate tubes, and the corresponding products pooled prior to the purification step preceding sequencing.

Input buffer
Input DNA must be eluted or resuspended 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.

B. 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.

C. 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/sec–5°C/sec; 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 (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 D 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.D).

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

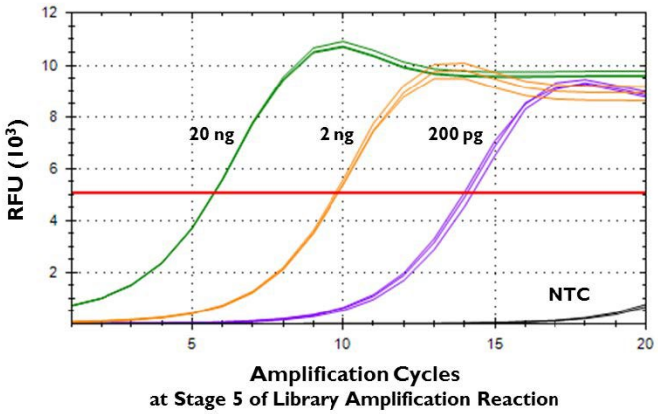

Figure 4. Example of real-time analysis of library amplification using ThruPLEX DNA-Seq. A typical real-time amplification analysis of libraries prepared with the ThruPLEX DNA-Seq Kit using 20 ng, 2 ng, or 200 pg of Covaris-sheared human DNA (GM 10851, Coriell Institute, 200 bp) relative to a No Template 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 5 of the Library Amplification Reaction (Section V.C). It is recommended to stay within one cycle above or below the optimal number of cycles. For example, for a 2 ng input, the optimal number of amplification cycles is 10 ± 1 cycles or 9 to 11 cycles. The Relative Fluorescence Unit (RFU) values on the y-axis may vary based on the instrument used.

D. Positive and Negative Controls

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

A suitable positive control (reference DNA) is Covaris-sheared purified genomic DNA (200–300 bp) of comparable input amount. Always prepare fresh dilutions of reference DNA. 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.

E. 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.

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).

F. 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 DNA-Seq library preparation protocol (Section V), 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. 1000000041074-v8).

G. Target Enrichment

ThruPLEX DNA-Seq is compatible with the major exome and target enrichment products, including Agilent SureSelectXT, XT2 and QXT, Roche NimbleGen SeqCap EZ, IDT xGEN Lockdown Probes, and the Illumina Nextera Rapid Capture Exome Enrichment Kit. ThruPLEX DNA-Seq target enrichment protocols can be accessed through the Learning Center at takarabio.com.

V. Protocols

A. Protocol: Template Preparation

Template Preparation Reagents

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

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 10 µl of fragmented doubled-stranded DNA into each PCR tube or well of a PCR plate.
   - **Positive control reactions using reference DNA:** If necessary, assemble reactions using 10 µl of a reference gDNA (e.g., Covaris-fragmented DNA, 200–300 bp average size) at an input amount comparable to the samples.
   - **Negative control reactions/No Template Controls (NTCs):** If necessary, assemble NTCs with 10 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 10 µl.

2. Prepare the template preparation D master mix as outlined below for the desired number of reactions, plus 5% of the total volume to allow for pipetting losses.

   Template preparation D master mix:

2 µl	Template Preparation D Buffer
1 µl	Template Preparation D Enzyme
3 µl	**Total volume per reaction**

   Mix thoroughly with a pipette. Keep on ice until used.

3. Assemble the template preparation reaction mixture as shown below. To each 10 µl sample from Step 1 above, add 3 µl of the template preparation D master mix.

Template preparation reaction mixture:

10 µl	Sample
3 µl	Template preparation D master mix (From Step 2)
13 µl	**Total volume per reaction**

4. Mix thoroughly with a pipette. Avoid introducing 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.
7. Place the plate or tube(s) in a thermal cycler with heated lid set to 101–105°C. Perform the template preparation reaction using the following conditions:

22°C	25 min
55°C	20 min
4°C	Hold for ≤2 hr

8. After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly.
9. Continue to library synthesis (Section V.B) in the same plate or tube(s).

B. Protocol: Library Synthesis

Library Synthesis Reagents

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

1. Prepare library synthesis D master mix as outlined below for the desired number of reactions, plus 5% of the total volume to allow for pipetting losses.

Library synthesis D master mix:

1 µl	Library Synthesis D Buffer
1 µl	Library Synthesis D Enzyme
2 µl	**Total volume per reaction**

Mix thoroughly with a pipette. Keep on ice until used.

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

Library synthesis reaction mixture:

13 µl	Template preparation reaction product (From Section V.A)
2 µl	Library synthesis D master mix (From Step 1)
15 µl	**Total volume per reaction**

Mix thoroughly with a pipette.

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 following conditions:

22°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.
8. Continue to Section V.C, "Protocol: Library Amplification", in the same plate or tube(s) maintained at 4°C.

C. Protocol: Library Amplification

Multiple stages occur during the library amplification reaction (see PCR program in Step 6, 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 (sold separately, see Tables 2–4 in Section III) 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 input DNA used. 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 5):
The number of PCR cycles required at Stage 5 of the library amplification reaction is dependent on the amount of input DNA and thermal cycler 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 300–700 ng library**
50 ng	6–8
20 ng	7–8
10 ng	7–8
5 ng	7–9
2 ng	8–10
1 ng	11–12
0.2 ng	14–15
0.05 ng	15–16

- **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 a 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 300–700 ng.

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

Library Amplification Reagents

Reagent	Cap Color
Library Amplification D Buffer	Green
Library Amplification Enzyme	Green
Nuclease-Free Water	Clear
Fluorescent dyes (optional)	
Indexing reagents (sold separately)	

Indexing reagents are available from Takara Bio in plate-based formats at a concentration of 12.5 µM per primer pair (Unique Dual Index Kit, Cat. Nos. 634752–634756) and in tube-based formats at a concentration of 5 µM per primer pair (DNA Single Index Kit, Cat Nos. R400695 & R400697; DNA Unique Dual Index Kit, Cat. Nos. R400665–R400668). All indexing reagents have been validated for use with the ThruPLEX DNA-Seq Kit, but the volume of reagent used per reaction varies depending on the indexing format used, as described below.

1. Prepare the indexing reagents by removing the from the freezer and thawing for 10 min on the bench top.

   Mix and spin the indexing reagents in a tabletop centrifuge to collect contents at the bottom of the well.

2. Prepare library amplification D master mix as outlined below for the desired number of reactions, plus 5% of the total volume to allow for pipetting losses. Mix thoroughly with a pipette. Keep on ice until used.

   Library amplification D master mix:

25 µl	Library Amplification D Buffer
1 µl	Library Amplification Enzyme
4 µl	Nuclease-Free Water (or fluorescent dyes)*
30 µl	**Total volume per reaction**

   *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.

   - **If monitoring in real-time:** The volume of detection and calibration dyes plus nuclease-free water should not exceed 4 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 and 1.5 µl of nuclease-free water per reaction to prepare the library amplification D master mix.

   - **If not monitoring in real-time:** If a regular thermal cycler is used, there is no need to add the dyes; use 4 µl of nuclease-free water per reaction in the library amplification D master mix.

3. Remove the seal on the PCR plate or open the tube(s).

4. Add 30 µl of the library amplification D master mix, the appropriate volume of indexing primers, and Nuclease-Free Water (only for the Unique Dual Index Kit) to each well or tube in the order listed. Mix thoroughly with a pipette but avoid introducing excessive air bubbles.

   IMPORTANT: Follow the volumes specific to the indexing kit being used.

   - If using a **Unique Dual Index Kit** (Cat. Nos. 634752–634756), combine:

15 µl	Library Synthesis Reaction Product (from Section V.B)
30 µl	Library amplification D master mix (from Step 2)
2 µl	Unique Dual Index Kit primers
3 µl	Nuclease-Free Water
50 µl	**Total volume per reaction**

     Recommendations for using the Unique Dual Index Kit:

     a. Thoroughly wipe the foil seal with 70% ethanol and allow it to dry to prevent cross-contamination.
     b. Make sure that the barcode label on the long side of the index plate is facing you.
     c. Use a clean pipette tip to pierce the seal above the specific indexing reagent on the plate; discard the tip used for piercing.
     d. Use a clean pipette tip to collect the indexing reagent and add to the reaction mixture.

   - If using a **DNA Unique Dual Index Kit** (Cat. Nos. R400665–R400668) or **DNA Single Index Kit** (Cat. Nos. R400695 & R400697), combine:

15 µl	Library Synthesis Reaction Product (from Section V.B)
30 µl	Library amplification D master mix (from Step 2)
5 µl	DNA Unique Dual Index Kit ***-OR-*** DNA Single Index Kit primers
50 µl	**Total volume per reaction**

5. 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.

6. 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 following conditions:

   CAUTION: Ensure 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**
4 cycles:			
98°C	20 sec	**4**	**Addition of indexes**
67°C	20 sec		
72°C	40 sec		
6–16 cycles*:			
98°C	20 sec	**5**	**Library Amplification**
72°C†	50 sec		
4°C	Hold	**6**	

*See Table 6, "▲ Stage 5 Amplification Guide", on the next page.
†If monitoring amplification in real-time, acquire fluorescence data at this step.

Table 6. ▲ Stage 5 amplification guide.

Input DNA	Number of cycles required to generate 300–700 ng library
50 ng	6–8
20 ng	7–8
10 ng	7–8
5 ng	7–9
2 ng	8–10
1 ng	11–12
0.2 ng	14–15
0.05 ng	15–16

7. Remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents at the bottom of each well. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to Section V.D.

SAFE STOPPING POINT: Samples can be processed immediately or stored frozen at –20°C for later processing.

D. Library Processing for Illumina Next Generation Sequencing

This section contains guidelines for processing ThruPLEX DNA-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 to. For more information, contact technical support.

Libraries prepared from each sample will contain the specific indexes selected at the time of the amplification. Follow the recommended workflow (solid arrows) in Figure 5 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, then an equal volume of each individual uniquely indexed library 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.

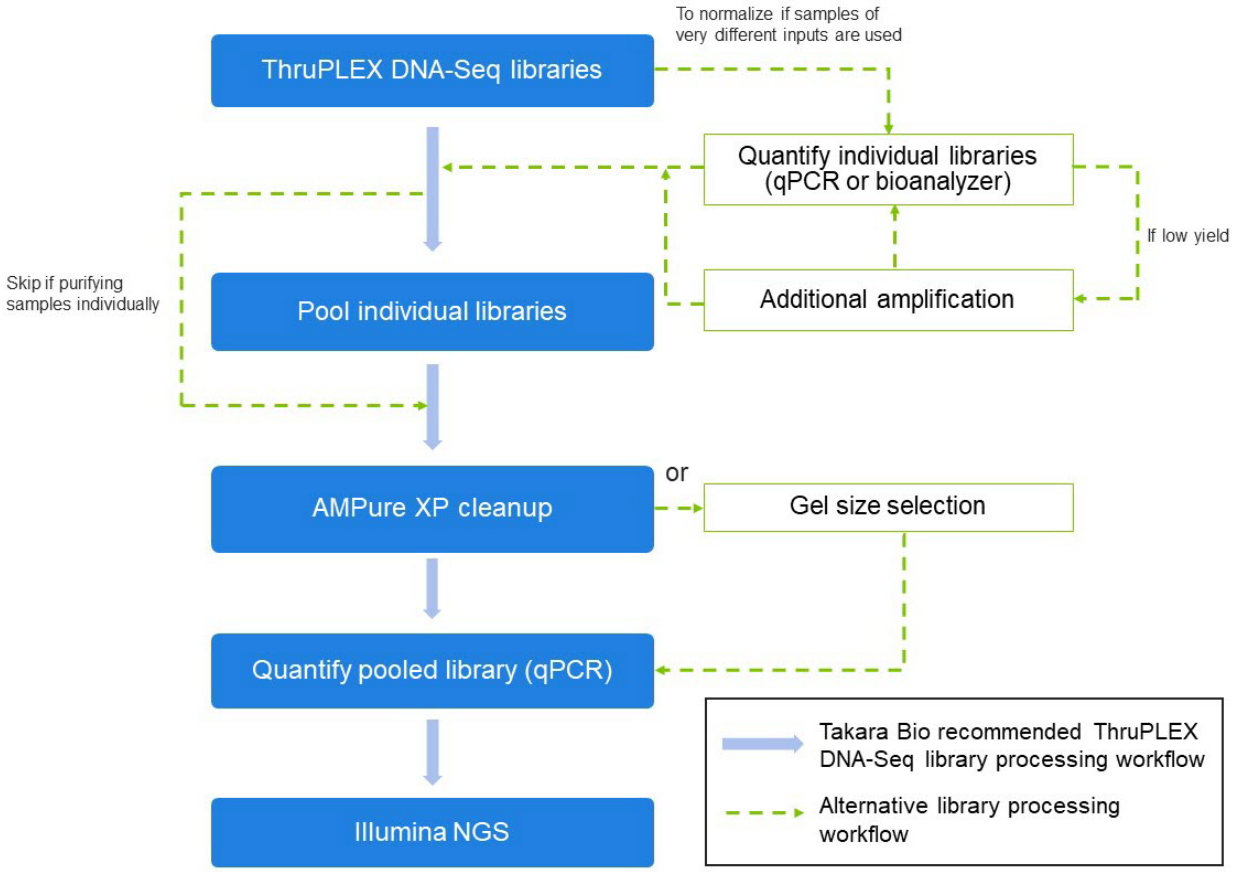

Figure 5. Workflow for processing the ThruPLEX DNA-Seq amplified libraries for Illumina NGS.

1. Library Quantification

There are several approaches available for library quantification, including real-time PCR, UV absorption, fluorescence detection, or 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 of sheared or fragmented starting gDNA inserts used for library construction.

UV absorption/fluorescence detection-based methods [i.e., Nanodrop (Thermo Scientific), Qubit 2.0 Fluorometer (Life Technologies), or Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies)] quantify total 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 the clustering competency.

Quantify ThruPLEX DNA-Seq library by real-time qPCR

Use the appropriate instrument-specific library quantification kit for Illumina NGS libraries as Takara Library Quantification Kit (Takara Bio, Cat. No. 638324). Dilute 2–5 µl of the library using a 100,000-fold dilution and use this as the template for quantification. Since the adapters result in an approximately 140 bp increase in the DNA fragment size, adjust the length accordingly to calculate the concentration of your library. For example, for a 200 bp DNA input,

and taking into account the distribution of fragment size, it is recommended to use 300 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 DNA-Seq library 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 following the manufacturer's instructions (Agilent Technologies, Cat. No. 5067-4626).

2. Additional Amplification

If the results show less than desirable yield, the remaining library can be further amplified to attain a higher yield (unless a plateau has been reached). The additional amplification can only be performed on **unpurified** libraries. ThruPLEX DNA-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:

1. If it was stored at –20°C after library amplification (Section V.C), thaw the plate or tube. Otherwise proceed directly to Step 2.
2. Spin down the tube(s) or plate containing the libraries.
3. Transfer the tube(s) or plate to a thermal cycler and perform 2–3 PCR cycles as follows:

2–3 cycles:		
	98°C	20 sec
	72°C	50 sec
4°C		Hold

3. Library Pooling for Purification

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

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 AM Pure XP purification purposes.

Illumina sequencers use a green laser to sequence G/T nucleotides and a red laser to sequence A/C nucleotides. At each sequencing cycle of the index read, at least one of the two nucleotides for each color channel should be present to ensure proper image registration and accurate demultiplexing of pooled samples. Color balance for each base is maintained by selecting index combinations that display this green/red channel diversity at each cycle. Please see Illumina Index Adapters Pooling Guide (1000000041074 v02) for guidelines on selecting the appropriate indexes for pooling and multiplexing.

4. Library Purification by AMPure XP beads

NOTE: AMPure XP purification is not necessary if gel size selection is performed.

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.

Library purification reagents (supplied by the user)

- AMPure XP beads
- Magnetic separator
- Freshly prepared 80% (v/v) ethanol
- TE buffer, pH 8.0 (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 vortexing 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 a 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).
5. Without disturbing the pellet, use a pipette to remove and discard the supernatant. 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, pH 8.0; 0.1 mM EDTA) 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.

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

5. Library Purification by Gel Size Selection (Alternate)

NOTE: Gel size selection is not necessary if AMPure XP purification is performed.

ThruPLEX DNA-Seq libraries can be size-selected prior to sequencing using agarose gel electrophoresis as described in the Illumina Paired-End Sample Preparation Guide (Illumina, Part No. 1005063 Rev. E, 2011), Illumina TruSeq® DNA Sample Preparation Guide (Illumina, Part No. 15026486 Rev. C, 2012), or by using automated platforms such as LabChIP (Caliper Life Sciences), Pippin Prep (Sage Science), or a similar technology.

When using agarose gel electrophoresis, extraction of the DNA should be performed with QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28704), or MinElute Gel Extraction Kit (Qiagen, Cat. No. 28604) following the manufacturer's instructions.

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

6. Sequencing Recommendations

The ThruPLEX DNA-Seq Kit generates libraries which 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 DNA-Seq Kit result in a size distribution of library fragments that is dependent on the input DNA fragment size (Figure 6).

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 of ThruPLEX DNA-Seq libraries. For sequencing on the Illumina MiSeq®, v3, load 14–15 pM of ThruPLEX DNA-Seq libraries with an average size of 300 bp.

Illumina recommends adding 1 % PhiX control for most libraries. PhiX is a small genome that can be quickly aligned to calculate error rates. It provides a balanced and diverse library to prevent sequencing problems. 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, 2016). 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).

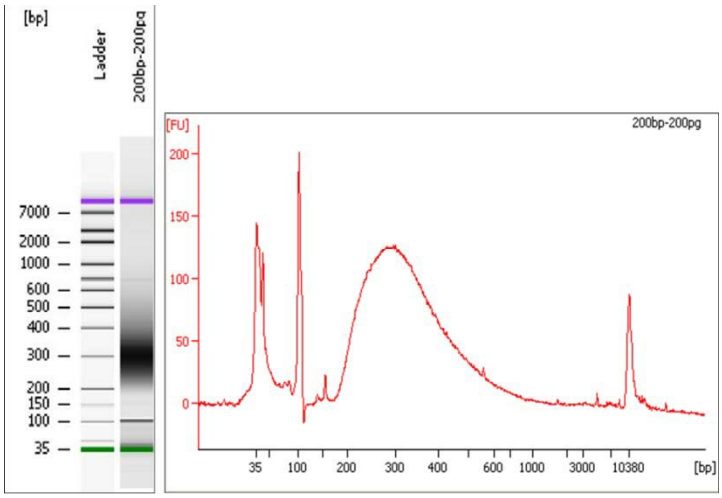

Figure 6. Bioanalyzer analysis of libraries prepared using ThruPLEX DNA-Seq. Libraries were prepared from 200-pg DNA (200 bp) using the ThruPLEX DNA-Seq Kit. Following library amplification, an aliquot of each library was diluted 1:4 in TE buffer, and 1 µl of this diluted sample was loaded onto a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies). A subsequent AMPure XP purification step would remove fragments around and below 100 bp.

Appendix A. Troubleshooting Guide

Table 7. Troubleshooting guide for the ThruPLEX DNA-Seq Kits.

Problem	Potential Cause	Suggested Solutions
Sample amplification curve looks like No Template Control (NTC) amplification curve or does not produce amplified product	No input DNA added	Quantitate input before using the kit
	Incorrect library template used (e.g., RNA, ssDNA)	Adhere to DNA Sample Requirements (Section III.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 multiple peaks in addition to the markers	Input sample contains unevenly fragmented DNA of various sizes (e.g., plasma DNA)	If possible, quantify and check input DNA prior to using the kit. Sequencing is still recommended.
After purification of the amplified library, Bioanalyzer traces show broad peak(s) extending from less than 1,000 bp to greater than 1,000 bp	Library is over-amplified or Bioanalyzer chip is 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

Problem	Potential Cause	Suggested Solutions
Low yield (continued)	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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