RUBICON GENOMICS

ThruPLEX® DNA-seq Kit Instruction Manual

High Performance Library Preparation for Illumina® NGS Platforms

Research Use Only

Product Use Limitations

ThruPLEX® DNA-seq Kit is intended for **Research Use Only**. It may not be used for any other purposes including, but not limited to, use in diagnostics, forensics, therapeutics, or in humans. ThruPLEX DNA-seq may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products without prior written approval of Rubicon Genomics, Inc.

***Protected by U.S. Patents 7,803,550; 8,071,312; 8,399,199; 8,728,737 and corresponding foreign patents. Additional patents are pending.**

Contents

Product Description

ThruPLEX® DNA-seq builds on the innovative ThruPLEX chemistry to generate DNA libraries with expanded multiplexing capability and with even greater diversity. Kits contain up to 96 Illumina-compatible indexes. ThruPLEX DNA-seq can be used in DNA-seq, RNA-seq, or ChIP-seq and offers robust target enrichment performance with all of the leading platforms. For more information, please visit: http://rubicongenomics.com/products/thruplex-dna-seq-kit/.

Kit Contents

ThruPLEX DNA-seq Kit contains sufficient reagents to prepare up to the specified number of reactions. Enough buffers and enzymes are provided for 4 uses or freeze-thaw cycles. Contents of ThruPLEX DNA-seq Kit are not interchangeable with other Rubicon Genomics products.

Table 1: ThruPLEX DNA-seq Kit Contents – Single Index Kits

Name	Cap Color	6S Kit (12 Rxn) R400523 6 Single Indexes 12 Reactions	12S Kit (48 Rxn) R400428 12 Single Indexes 48 Reactions	48S Kit R400427 48 Single Indexes 48 Reactions
Template Preparation Buffer	Red	1 Tube	1 Tube	1 Tube
Template Preparation Enzyme	Red	1 Tube	1 Tube	1 Tube
Library Synthesis Buffer	Yellow	1 Tube	1 Tube	1 Tube
Library Synthesis Enzyme	Yellow	1 Tube	1 Tube	1 Tube
Library Amplification Buffer	Green	1 Tube	1 Tube	1 Tube
Library Amplification Enzyme	Green	1 Tube	1 Tube	1 Tube
Nuclease-Free Water	Clear	1 Tube	1 Tube	1 Tube
Indexing Reagents	Blue	6 Tubes	12 Tubes	1 Single Index Plate (48S)
Quick Protocol				

Table 2: ThruPLEX DNA-seq Kit Contents – Dual Index Kits

Name	Cap Color	48D Kit R400406 48 Dual Indexes 48 Reactions	96D Kit R400407 96 Dual Indexes 96 Reactions
Template Preparation Buffer	Red	1 Tube	2 Tubes
Template Preparation Enzyme	Red	1 Tube	2 Tubes
Library Synthesis Buffer	Yellow	1 Tube	2 Tubes
Library Synthesis Enzyme	Yellow	1 Tube	2 Tubes
Library Amplification Buffer	Green	1 Tube	2 Tubes
Library Amplification Enzyme	Green	1 Tube	2 Tubes
Nuclease-Free Water	Clear	1 Tube	1 Tube
Indexing Reagents		1 Dual Index Plate (48B or 48D)	1 Dual Index Plate (96A or 96D)
Quick Protocol			

- **Note:** Dual Index Plates (48B) and (48D) are identical; Dual Index Plates (96A) and (96D) are also identical.

Shipping and Storage

ThruPLEX DNA-seq Kit is shipped on dry ice. The kit should be stored at −20°C upon arrival.

Quality Control

ThruPLEX DNA-seq Kit is functionally tested using Next Generation Sequencing (NGS) to ensure product quality and consistency.

Safety Information

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 http://rubicongenomics.com/resources/msds/.

Technical Assistance

For technical support with any of the Rubicon Genomics, Inc. products please contact technical support by email: support@rubicongenomics.com or call at (734)-677-4845 (9 AM – 5:30 PM EST).

A. Introduction

Next Generation Sequencing (NGS) is a dynamic field with rapidly evolving needs. Regardless of sample type or application, a DNA library must be prepared from each sample in order to be sequenced on Illumina NGS platforms. The process of library preparation (Figure 1) involves placing Illumina sequencing adapters on DNA fragments and adding Illumina-compatible indexes to allow pooling of multiple samples (multiplexing). This library preparation is a critical step in the NGS workflow and has direct impact on the quality of sequencing results.

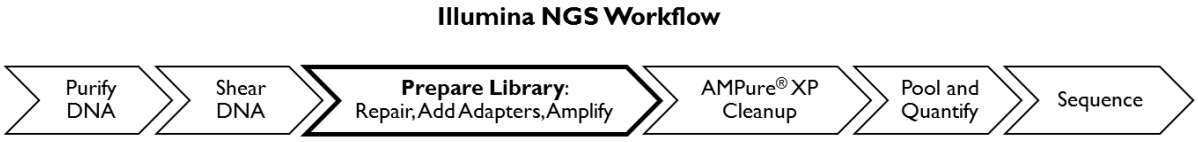

Figure 1. Illumina NGS workflow: DNA samples are first purified and sheared. Library preparation follows, consisting of repair, Illumina adapter addition, and DNA fragment amplification. Indexed libraries are purified, pooled, and quantified prior to sequencing on Illumina NGS platforms.

As NGS clinical applications emerge and as NGS instruments become more powerful, researchers and clinicians are increasingly investigating more difficult and challenging samples which are present in limited quantities, used in small amounts, and/or damaged. In many applications, such as cell-free DNA from plasma, the sample DNA material is limited and highly degraded. In cases where DNA is not limited, such as analysis of tumor tissues, the ability to use low input amounts is important for conserving samples for multiple uses. Clinical samples also necessitate careful tracking of samples; a protocol in which the sample never leaves the tube is advantageous to ensure accurate sample tracking and to avoid contamination. This growing trend requires library preparation kits which accurately preserve the complexity of the samples and provide higher sensitivity and greater multiplexing capability, with a simple workflow.

The commitment to fulfill these needs is the core of ThruPLEX DNA-seq. It has been developed to expand multiplexing capability and provide high-quality, Illumina-compatible NGS libraries from low input amounts. ThruPLEX DNA-seq's three-step, single-tube library preparation workflow (Figure 2) is the simplest in the industry and minimizes handling errors and loss of valuable samples.

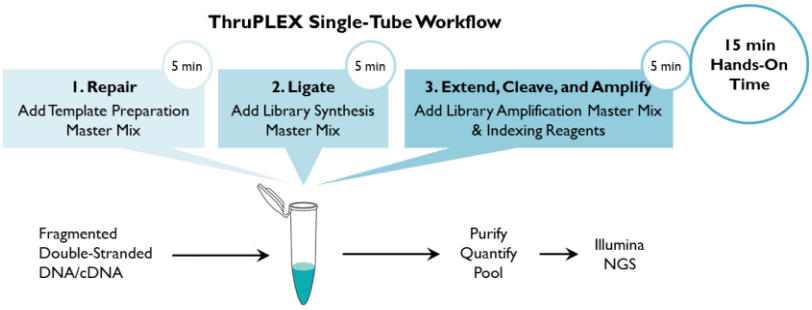

Figure 2. ThruPLEX DNA-seq single-tube library preparation workflow: The ThruPLEX DNA-seq workflow consists of 3 simple steps that take place in the same PCR tube or well and eliminates the need to purify and transfer the sample material.

B. ThruPLEX DNA-seq Kit

I. Overview

The ThruPLEX DNA-seq Kit is designed to provide up to 96 indexed libraries for higher multiplexing capabilities on Illumina NGS platforms. The 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 includes all necessary reagents including indexes for multiplexing up to 96 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 for use with small fragments of DNA such as cell-free plasma DNA.

II. Principle

The ThruPLEX DNA-seq Kit is based on Rubicon Genomics' 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 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.

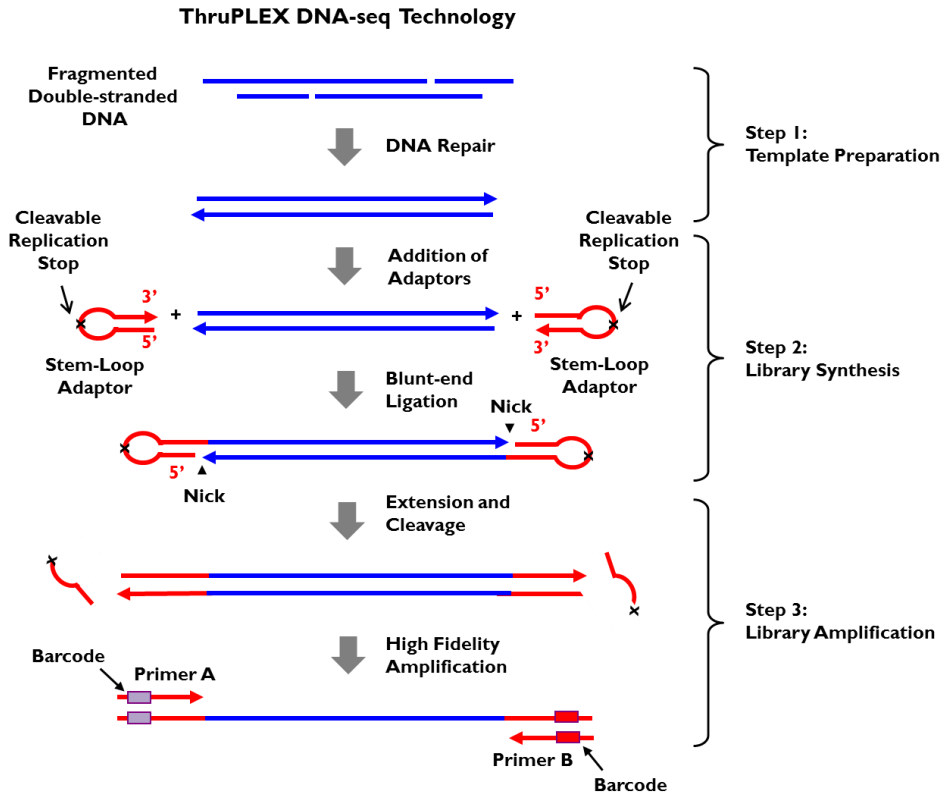

Figure 3. ThruPLEX DNA-seq technology: A three-step, single-tube reaction that starts with fragmented double-stranded DNA (0.05 ng to 50 ng). Stem-loop adapters are blunt end ligated to repaired input DNA. These molecules are extended then amplified to include barcodes using a high fidelity polymerase to yield an indexed Illumina NGS library.

III. ThruPLEX DNA-seq Workflow

The ThruPLEX DNA-seq Kit workflow is highly streamlined (Figure 4) and consists of the following three steps:

- **Template Preparation** for efficient repair of the fragmented double-stranded DNA input.
- **Library Synthesis** for ligation of Rubicon Genomics' 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.

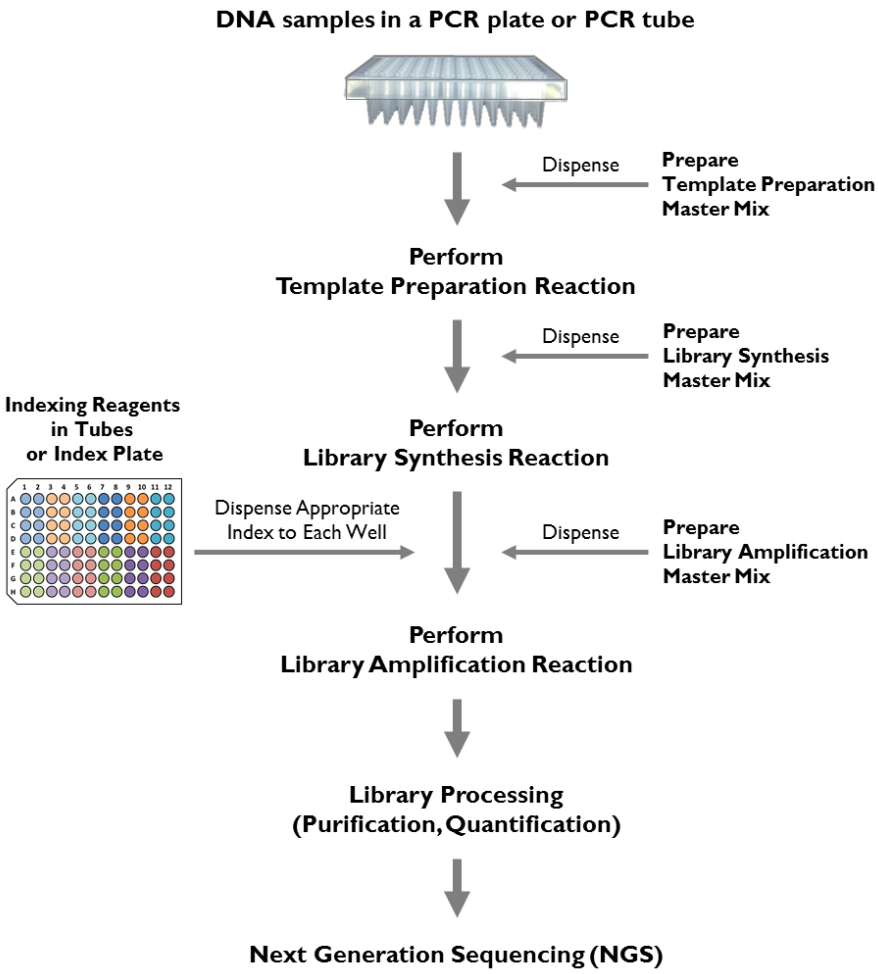

Figure 4. ThruPLEX DNA-seq library preparation workflow overview: Steps involved in ThruPLEX library preparation for Illumina NGS starting with fragmented DNA.

C. Getting Started

I. Additional Supplies and Equipment Needed

Required Supplies and Equipment

- Thermal cycler (real-time instrument recommended)

Note: See Thermal Cycler Considerations below.

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

- Low binding aerosol barrier tips
- Freshly prepared 80% (v/v) ethanol
- Agencourt® AMPure® XP beads (Beckman Coulter, CAT. NO. A63880)

Optional Supplies

- KAPA® Library Quantification Kit – Illumina (Kapa Biosystems, CAT. NO. specific to real time PCR system used)
- EvaGreen® Dye, 20X in water (Biotium, CAT. NO. 31000-T)
- Fluorescein Calibration Dye (Bio-Rad Laboratories, CAT. NO. 170-8780)

II. 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 Supplies above) to the reaction (Figure 5). 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 E.II**.).

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 D.III.**). For some real-time instruments, calibration dye may not be needed; please refer to the real-time thermal cycler instrument's manual.

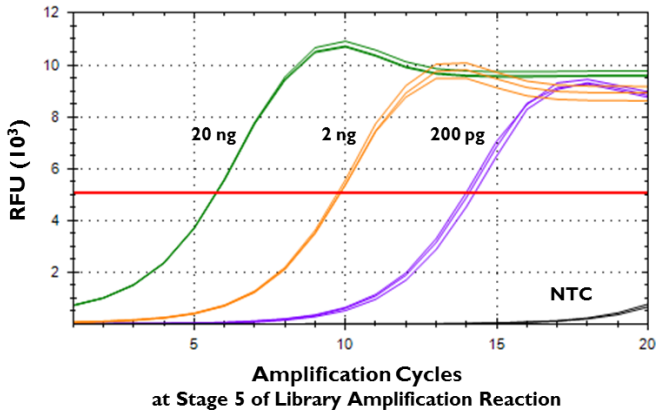

Figure 5. Example of real-time analysis of library amplification using ThruPLEX DNA-seq: A typical real-time amplification analysis of libraries prepared with ThruPLEX DNA-seq Kit using 20 ng, 2 ng, or 200 pg of Covaris-sheared human DNA (GM 10851, Coriell Institute, 200 bp) relative to a No Template Control (NTC). Results were obtained using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) 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 D.III.**). 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.

III. Starting Material

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.

IV. Positive and Negative Controls

If necessary, include appropriate positive and negative controls in the experimental design to help verify that reactions proceed as expected. If the experimental samples contain any carryover contaminant(s) in the buffer, the downstream reactions may be impacted, and inclusion of controls would help elucidate such problems. 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.

V. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared at each workflow step based on the number of reactions to be performed. Transfer the enzymes to ice just prior to use and centrifuge briefly to collect contents at the bottom of the tube. Thaw the buffers, vortex briefly and centrifuge prior to use. Keep all the components and master mixes on ice. Once the master mix is

prepared, thoroughly mix the contents several times with a pipette while avoiding introduction of excessive air bubbles and briefly centrifuge prior to dispensing into the PCR plate or tube(s).

VI. Indexing Reagents

ThruPLEX DNA-seq Kit includes all necessary reagents including Indexing Reagents for multiplexing samples. The Indexing Reagents consist of amplification primers containing Illumina-compatible indexes. Index sequences can be downloaded as CSV files at the ThruPLEX DNA-seq Product Page, under the Resources tab: http://rubicongenomics.com/products/thruplex-dna-seq-kit/.

Before starting the ThruPLEX DNA-seq Library Preparation Protocol (**Section D**), refer to **Appendix I** for information on index sequences, Index Plate handling instructions, and multiplexing and index pooling guidelines.

ThruPLEX DNA-seq 6S (12 Rxn) Kits

Indexing Reagents are pre-dispensed in 6 individual tubes with blue caps. Each tube contains sufficient volume for up to 8 uses. No more than 4 freeze/thaw cycles are recommended for the Indexing Reagent Tubes.

ThruPLEX DNA-seq 12S (48 Rxn) Kits

Indexing Reagents are pre-dispensed in 12 individual tubes with blue caps. Each tube contains sufficient volume for up to 8 uses. No more than 4 freeze/thaw cycles are recommended for the Indexing Reagent Tubes.

ThruPLEX DNA-seq 48S, 48D, and 96D Kits

Indexing Reagents are pre-dispensed and sealed in a linear barcoded Index Plate. The Index Plate is sealed with foil that can be pierced with a multichannel pipet tip to collect the required amount of index to assemble the reactions. Each well of the Index Plate contains sufficient volume for a single use. No more than 4 freeze/thaw cycles are recommended for the Index Plate.

VII. Using Illumina Experiment Manager

Make sure the latest version of the Illumina Experiment Manager (IEM) is installed (version 1.8 or later). Prior to starting the ThruPLEX DNA-seq Library Preparation Protocol (**Section D**), create a Sample Sheet in the IEM to select and validate appropriate indexes to use in your experiments. Refer to **Appendix 1** for guidelines on using the IEM to validate your index combinations.

VIII. Target Enrichment

ThruPLEX DNA-seq is compatible with the major exome and target enrichment products, including Agilent SureSelect®, Roche NimbleGen® SeqCap® EZ and custom panels. ThruPLEX DNA-seq target enrichment protocols and application notes can be assessed through the Applications section of the Rubicon Genomics website at: http://rubicongenomics.com/applications/enrichment/.

D. ThruPLEX DNA-seq Library Preparation Protocol

I. Template Preparation Step

Template Preparation Reagents

Template Preparation Reagents	
Reagent	**Cap color**
Template Preparation Buffer	Red
Template Preparation Enzyme	Red

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

Template Preparation Protocol

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

Template Preparation Master Mix		
Component	**Cap color**	**Volume/Reaction**
Template Preparation Buffer	Red	2.0 µL
Template Preparation Enzyme	Red	1.0 µL

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

Template Preparation Reaction Mixture	
Component	**Volume/Reaction**
Sample	10 µL
Template Preparation Master Mix	3 µL
Total Volume	13 µL

4. Mix thoroughly with a pipette.
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°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
4°C	Hold for ≤ 2 hours

8. After the thermal cycler reaches 4°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).

II. Library Synthesis Step

Library Synthesis Reagents

Library Synthesis Reagents	
Reagent	**Cap Color**
Library Synthesis Buffer	Yellow
Library Synthesis 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.

Library Synthesis Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Library Synthesis Buffer	Yellow	1.0 µL
Library Synthesis Enzyme	Yellow	1.0 µL

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

3. Assemble the **Library Synthesis Reaction Mixture** as shown in the table below. To each well or tube, add 2 µL of the **Library Synthesis Master Mi**x.

Library Synthesis Reaction Mixture	
Component	**Volume/Reaction**
Template Preparation Reaction Product	13 µL
Library Synthesis Master Mix	2 µL
Total Volume	15 µL

4. Mix thoroughly with a pipette.
5. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
6. Centrifuge briefly to collect the contents to the bottom of each well or tube.
7. Return the plate or tube(s) to the thermal cycler with heated lid set to 101°C – 105°C. Perform **Library Synthesis Reaction** using the cycling conditions in the table below:

Library Synthesis Reaction	
Temperature	**Time**
22°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.

III. Library Amplification Step

Multiple stages occur during the **Library Amplification Reaction** (see table in step 8 below). Stage 1 and Stage 2 extend and cleave the stem loop adapters. **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 4 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.

- **Note:** Refer to **Appendix 1** for selecting the appropriate indexes to use for your experiments.

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	
DNA Input (ng)	**Number of Cycles**
50	5
20	6
10	7
5	8
2	10
1	11
0.2	14
0.05	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 plateau. Determine the optimal number of amplification cycles by constructing PCR curves and identifying the midpoint of the linear phase as illustrated in Figure 5. Use the optimal amplification cycle number in the actual experiment for sequencing.
- **Yield:** The amount of amplified library can range from 100 ng to 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 ng to 700 ng.
- **Note:** Over amplification could result in higher rate of PCR duplicates in the library.

Library Amplification Reagents

Library Amplification Reagents	
Reagent	**Cap Color**
Library Amplification Buffer	Green
Library Amplification Enzyme	Green
Nuclease-Free Water	Clear
Fluorescent Dyes	
Indexing Reagents	Tubes (Blue) or Index Plate

- **Note:** 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 **Appendix 1** for Index Plate handling instructions. No more than 4 freeze/thaw cycles are recommended for the Index Plate.

Library Amplification Protocol

1. Prepare the Indexing Reagents
    - Remove the Indexing Reagents from freezer and thaw for ten minutes on the bench top.
    - Spin the Indexing Reagents in a table top centrifuge to collect contents at the bottom of the well.

- Thoroughly wipe the Indexing Reagent Tubes or Index Plate foil seal with 70% ethanol and allow it to dry.

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

Library Amplification Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Library Amplification Buffer	Green	25.0 µL
Library Amplification Enzyme	Green	1.0 µL
Nuclease-Free Water (plus fluorescent dyes*)	Clear	4.0 µL

- **If monitoring in real-time:** Fluorescence dyes* (for detection and optical calibration) are added when monitoring amplification in real time during cycling. Please refer to the real-time PCR instrument's user manual for calibration dye recommendations. The volume of detection and calibration dyes plus nuclease-free water should not exceed 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 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 Master Mix.

3. Remove the seal on the PCR plate or open the tube(s)
4. Add 30 µL of the **Library Amplification Master Mix** to each well or tube.
5. Add 5 µL of the appropriate Indexing Reagent to each well or tube:

Library Amplification Reaction Mixture	
Component	**Volume/Reaction**
Library Synthesis Reaction Product	15 µL
Library Amplification Master Mix	30 µL
Indexing Reagent	5 µL
Total Volume	50 µL

For ThruPLEX DNA-seq 48S, 48D, 96D Kits containing 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 pipet tip to pierce the seal above the specific Indexing Reagent on the Index Plate; discard the tip used for piercing.
- Use a clean pipet tip to collect 5 µL of the Indexing Reagent and add to the reaction mixture.
  - **Note:** Follow the Index Plate handling instructions in **Appendix 1** to avoid cross contamination.

6. Mix thoroughly with a pipette. Avoid introducing excessive air bubbles.

7. Seal the PCR plate or tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well or tube.

- **Note:** Use optical sealing tape if a real-time thermal cycler is used.

8. Return the plate or tube(s) to the real-time PCR thermal cycler/thermal cycler with 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.**

Library Amplification Reaction				
	Stage	**Temperature**	**Time**	**Number of 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 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	▲ 5	98°C	20 s	▲ 5 to16 (see table on right)
		*72°C	50 s	
	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**
50	5
20	6
10	7
5	8
2	10
1	11
0.2	14
0.05	16

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.

- **Note:** At this stage, samples can be processed for next generation sequencing (NGS) immediately or stored frozen at –20°C for later processing. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to **Section E**.

E. Library Processing for Illumina Next Generation Sequencing

I. Overview

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 are given. For more information, contact technical support at support@rubicongenomics.com.

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 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, 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 flowcell. Instructions and recommendations on library pooling, purification, quantification, and sequencing are described in the following sections.

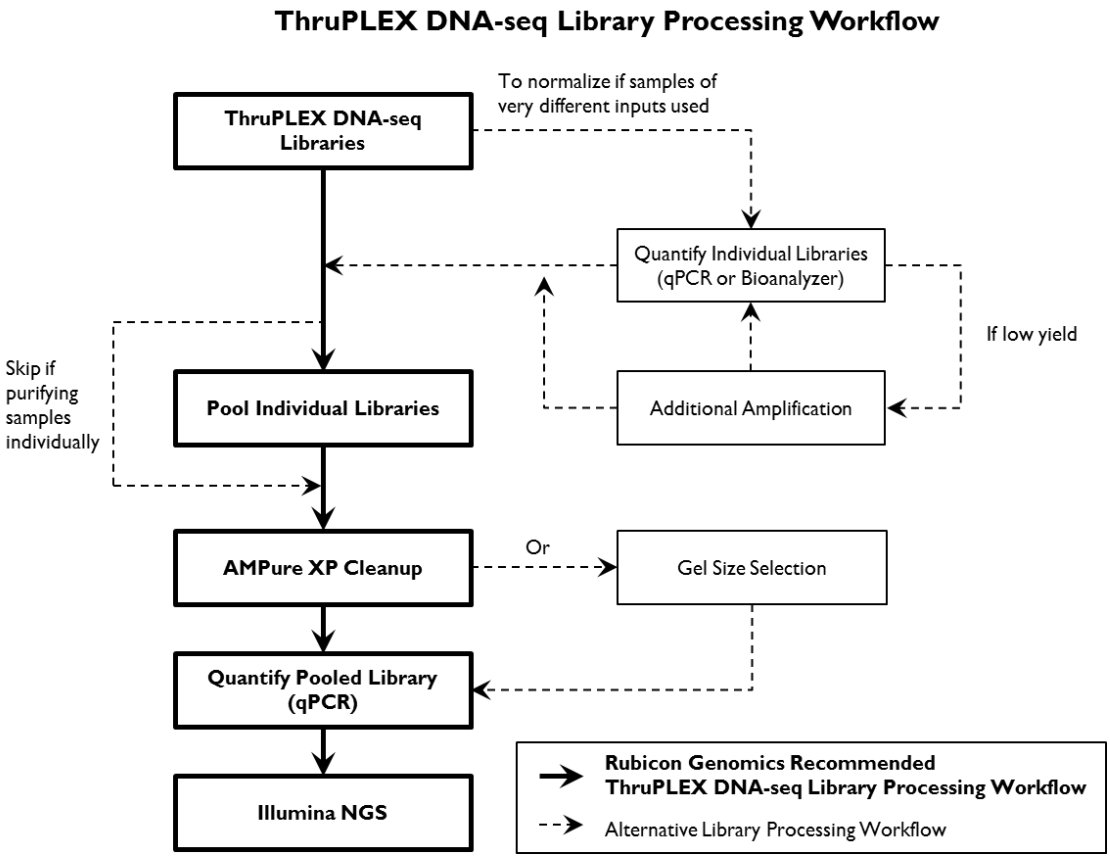

Figure 6. Workflow for processing the ThruPLEX DNA-seq amplified libraries for Illumina NGS.

II. 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 (such as the KAPA Library Quantification Kit from Kapa Biosystems) 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.

On the other hand, 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)) simply 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 about the clustering competency.

Quantify ThruPLEX DNA-seq library by real-time qPCR

Use the appropriate instrument-specific KAPA Library Quantification Kit for Illumina Sequencing Platforms (Kapa Biosystems). 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

Remove an aliquot of each library and dilute as appropriate in TE buffer. Load a 1µL aliquot of this diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, CAT. NO. 5067-4626).

III. 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, spin down a tube or plate containing the library, 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 s
	72°C	50 s
1 cycle	4°C	Hold

IV. 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 AMPure 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 **Appendix I** for guidelines on selecting the appropriate indexes for pooling and multiplexing.

V. 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)

Library Purification Reagents
AMPure XP beads
Magnetic rack for 1.5 mL centrifuge tubes
Freshly prepared 80% (v/v) ethanol
TE buffer, pH 8.0

AMPure XP Protocol

- **Note:**
  - It is important to bring all the samples and reagents to be used to room temperature.
  - Always use freshly prepared 80% (v/v) ethanol for Step 3 and Step 4 below.
  - Resuspend the AMPure XP reagent by gentle vortexing until no visible pellet is present at the bottom of the container.

AMPure XP Protocol (Continued)

1. In a 1.5 mL tube, mix 100 µL of AMPure XP reagent with a 100 µL aliquot of the pooled library ensuring a 1:1 (v/v) ratio. Mix by pipette 10 times to achieve a homogeneous solution and incubate the tube at room temperature for 5 min.
2. Pulse-spin the sample(s) on a bench top centrifuge and place the tube in a magnetic stand. Wait for 2 min or until the beads are completely bound to the side of the tube(s) and the solution is clear.
3. With the tube(s) in the magnetic stand and without disturbing the pellet use a pipette to aspirate off and discard the supernatant. Add 300 µL of 80% (v/v) ethanol to the pellet.
4. With the tube(s) in the magnetic stand, rotate each tube 90 degrees; wait until all the beads come to a halt. (DO NOT INVERT TUBE-RACK). Repeat this step three more times. Without disturbing the pellet, use a pipette to aspirate off and discard the supernatant. Add 300 µL of 80% (v/v) ethanol to the pellet.
5. With the tube(s) in the magnetic stand and without disturbing the pellet, turn each tube 90 degrees and wait until all the beads come to a halt. (DO NOT INVERT TUBE-RACK). Repeat this step three more times. Without disturbing the pellet, use a pipette to aspirate off and discard the supernatant.
6. Pulse-spin the sample(s) using a low speed, bench top centrifuge, place into a magnetic stand, and wait for 2 minutes or until the beads are completely bound to the side of the tube(s). With the tube(s) in the magnetic stand, use a pipette to aspirate off and discard any residual ethanol without disturbing the pellet.
7. Leaving the cap open, incubate the sample(s) in a heating block at 37°C for 2 – 3 min or until the pellet is dry. DO NOT OVER DRY THE PELLET(S).
8. Elute the DNA by re-suspending the beads with 50 µL of 1x TE buffer, pH 8.0. Pulse-spin the sample(s) using a low speed, bench top centrifuge and place it into a magnetic stand and let the beads bind to the side of the tube(s) completely (for ~2 min) until the solution is clear.
9. While keeping the sample(s) in the magnetic stand, without disturbing the pellet, transfer the supernatant with a pipette into a new tube. If not used immediately, the purified library can be stored at –20 °C.

VI. 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 # 1005063 Rev. E, 2011), Illumina TruSeq® DNA Sample Preparation Guide (Illumina, Part # 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 base pair increase in the size of each library.

VII. 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 7). 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, 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. For low diversity libraries and if experiencing sequencing issues, increase the PhiX control spike-in to at least 5%. 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 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 # 15039740 Rev. D, 2013).

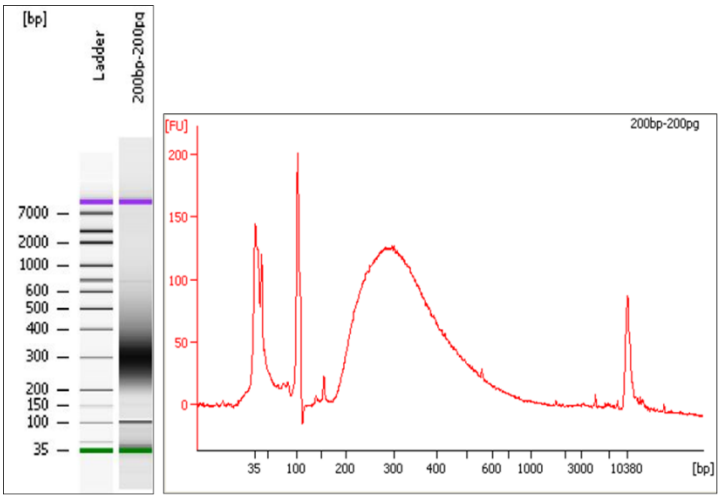

Figure 7. Bioanalyzer analysis of libraries prepared using ThruPLEX DNA-seq: Libraries were prepared from 200 pg DNA (200 bp) using the ThruPLEX DNA-seq Kit. Following library amplification, an aliquot of each library was diluted at 1:4 in TE buffer, and 1µL of this diluted sample was loaded onto a Bioanalyzer high

sensitivity DNA chip (Agilent Technologies). Subsequent AMPure XP purification step would remove fragments around and below 100 bp.

Appendix 1. Indexing Reagents

A. Overview

ThruPLEX DNA-seq Kits contain all necessary reagents to generate amplified and indexed NGS libraries, including Indexing Reagents for multiplexing up to 96 samples. Table 3 below summarizes the characteristics of the included Indexing Reagents, which consist of amplification primers containing Illumina-compatible indexes. Indexing Reagents should be stored at –20°C and should not be subjected to more than 4 freeze/thaw cycles.

- **Note:** Indexing Reagents provided with ThruPLEX DNA-seq Kit cannot be substituted with indexing reagents from any other sources.

Table 3: ThruPLEX DNA-seq Kit – Indexing Reagents

	6s Kit (12 Rxn)	12S Kit (48 Rxn)	48S Kit	48D Kit	96D Kit
Number of Reactions	12	48	48	48	96
Number of Indexes	6	12	48	48	96
Index Type	Single	Single	Single	Dual	Dual
Length of Indexes	8nt	8nt	8nt	8nt	8nt
Format	6 Tubes	12 Tubes	96-Well Plate	96-Well Plate	96-Well Plate
Number of Uses	Up to 8	Up to 8	Single	Single	Single
Illumina Experiment Manager Kit Selection	TruSeq LT or Manual Input	TruSeq LT or Manual Input	Manual Input only	TruSeq HT	TruSeq HT

When libraries with **less than the full set** of ThruPLEX DNA-seq indexes will be prepared and pooled, it is critical that compatible index combinations are used to fulfill Illumina requirements. 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 colored laser should be present to ensure proper image registration and ensure accurate demultiplexing of the pooled samples.

Follow the steps below **before** beginning the ThruPLEX DNA-seq Library Preparation Protocol if using less than the full set of indexes included with the kit:

1. Determine the number of libraries that will be pooled for sequencing.
2. Select the appropriate index combinations for multiplexing and pooling.

3. Use the Illumina Experiment Manager (IEM) to create a Sample Sheet which will be used during the sequencing run. The IEM can detect and warn of sub-optimal index combinations, allowing re-design **before** library preparation starts.

Appendices 1B to 1D provide index sequences, plate handling instructions, multiplexing and index pooling guidelines, and Sample Sheet setup instructions specific for each ThruPLEX DNA-seq Kit.

- For ThruPLEX DNA-seq 6S (12 Rxn) and 12S (48 Rxn) Kits, proceed to Appendix 1B
- For ThruPLEX DNA-seq 48S Kit, proceed to Appendix 1C
- For ThruPLEX DNA-seq 48D and 96D Kits, proceed to Appendix 1D

B. ThruPLEX DNA-seq 6S (12 Rxn) and 12S (48 Rxn) Kits

Single Index Sequences

ThruPLEX DNA-seq single indexes use Illumina-compatible 8nt sequences developed by the Wellcome Trust Sanger Institute in Cambridge, UK. Each Indexing Reagent Tube contains a unique single index sequence. The ThruPLEX DNA-seq 6S (12 Rxn) kits contain single indexes (Tubes 1-6) and share the same sequences in the first 6 bases as the Illumina TruSeq LT indexes AD001 through AD006. The 12 ThruPLEX DNA-seq single indexes share the same sequences in the first 6 bases as the Illumina TruSeq LT indexes AD001 through AD012 (Figure 8). The prepared library has the structure shown in Figure 9.

- **Note:** Information about the Sanger index sequences can be found in *Nature Methods* **7**, 111-118 (2010).

ThruPLEX DNA-seq Single Indexes				
Tube	**Sanger Tag**	**Sequence**	**TruSeq LT Index**	**TruSeq LT**
1	iPCRtagT1	**ATCACG**TT	AD001	ATCACG
2	iPCRtagT2	**CGATGT**TT	AD002	CGATGT
3	iPCRtagT3	**TTAGGC**AT	AD003	TTAGGC
4	iPCRtagT4	**TGACCA**CT	AD004	TGACCA
5	iPCRtagT5	**ACAGTG**GT	AD005	ACAGTG
6	iPCRtagT6	**GCCAAT**GT	AD006	GCCAAT
7	iPCRtagT7	**CAGATC**TG	AD007	CAGATC
8	iPCRtagT8	**ACTTGA**TG	AD008	ACTTGA
9	iPCRtagT9	**GATCAG**CG	AD009	GATCAG
10	iPCRtagT10	**TAGCTT**GT	AD010	TAGCTT
11	iPCRtagT11	**GGCTAC**AG	AD011	GGCTAC
12	iPCRtagT12	**CTTGTA**CT	AD012	CTTGTA

Figure 8. ThruPLEX DNA-seq Single Indexes: Each Indexing Reagent Tube contains a unique Illumina-compatible 8nt Sanger index sequence. The 6S (12 Rxn) kit contains single indexes (Tubes 1-6) that share the same sequence in the first 6 bases (show in BOLD) as the Illumina LT indexes AD001- AD006. The 12 ThruPLEX DNA-seq single indexes share the same sequence in the first 6 bases as the Illumina TruSeq LT indexes AD001 through AD012.

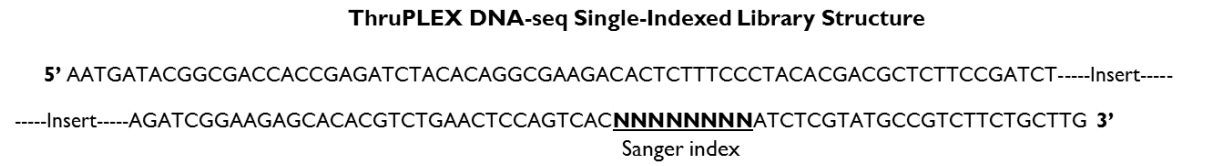

Figure 9. ThruPLEX DNA-seq single-indexed library structure: Libraries prepared from the ThruPLEX DNA-seq Kit contain the 8nt Sanger index sequence on the 3' end.

Multiplexing and Index Pooling

It is very important to select appropriate single indexes such that they are unique and meet the Illumina recommended compatibility requirements. For low-plex (2- to 11-plex) pooling guidelines, please refer to Illumina's TruSeq Sample Preparation Pooling Guide (Illumina, Document #15042173 v01).

- **Note:** For MiSeq RTA v1.17.28 and later, base pair diversity of indexes is no longer checked by IEM because low-plexity index reads can be processed for all applications; any combination of indexes can be pooled for sequencing on MiSeq.

Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool that allows you to create and edit Sample Sheets for Illumina sequencers. To use this tool with ThruPLEX indexes, ensure that the latest version of IEM (version 1.8 or later) is installed. There are two options for creating the Sample Sheet:

Option 1: In the IEM, on the "Workflow Parameters" page, select "TruSeq LT" in the dropdown menu for "Sample Prep Kit". Add indexes to be used on the "Sample Selection" page by clicking "Add Blank Row" and then choosing the appropriate indexes from the "index 1 (I7)" dropdown menu.

- **Note:** If TruSeq LT is selected, index combinations may be validated using the IEM; however, only the first 6 bases of the 8nt sequence will be sequenced.

Option 2: Manually copy and paste the appropriate 8nt single index sequences to be used to the CSV file of the Sample Sheet.

The 8nt single index sequences can be downloaded as a CSV file at the ThruPLEX DNA-seq Product Page, under the Resources tab: http://rubicongenomics.com/products/thruplex-dna-seq-kit/.

- **Note:** The IEM will not check for color-balanced index combinations when index sequences are entered manually from the CSV file.

C. ThruPLEX DNA-seq 48S Kit

Single Index Sequences

ThruPLEX DNA-seq single indexes use Illumina-compatible 8nt sequences developed by the Wellcome Trust Sanger Institute in Cambridge, UK. Each well of the Single Index Plate (SIP) contains a unique single index sequence (Figure 10). The first 12 ThruPLEX DNA-seq single indexes (wells A1 through A12) share the same sequences in the first 6 bases as the Illumina TruSeq LT indexes AD001

through AD012 (sequences provided in Appendix 1B). The prepared library has the structure shown in Figure 11.

- **Note:** Information about the Sanger index sequences can be found in *Nature Methods* **7**, 111-118 (2010).

ThruPLEX DNA-seq Single Indexes					
Well	**Sanger Tag**	**Sequence**	**Well**	**Sanger Tag**	**Sequence**
A1	iPCRtagT1	**ATCACG**TT	B1	iPCRtagT13	TGGTTGTT
A2	iPCRtagT2	**CGATGT**TT	B2	iPCRtagT14	TCTCGGTT
A3	iPCRtagT3	**TTAGGC**AT	B3	iPCRtagT15	TAAGCGTT
A4	iPCRtagT4	**TGACCA**CT	B4	iPCRtagT16	TCCGTCTT
A5	iPCRtagT5	**ACAGTG**GT	B5	iPCRtagT17	TGTACCTT
A6	iPCRtagT6	**GCCAAT**GT	B6	iPCRtagT18	TTCTGTGT
A7	iPCRtagT7	**CAGATC**TG	B7	iPCRtagT19	TCTGCTGT
A8	iPCRtagT8	**ACTTGA**TG	B8	iPCRtagT20	TTGGAGGT
A9	iPCRtagT9	**GATCAG**CG	B9	iPCRtagT21	TCGAGCGT
A10	iPCRtagT10	**TAGCTT**GT	B10	iPCRtagT22	TGATACGT
A11	iPCRtagT11	**GGCTAC**AG	B11	iPCRtagT99	GTGCTACC
A12	iPCRtagT12	**CTTGTA**CT	B12	iPCRtagT101	GGTTGGAC
Well	**Sanger Tag**	**Sequence**	**Well**	**Sanger Tag**	**Sequence**
C1	iPCRtagT25	TGCGATCT	D1	iPCRtagT102	GGCACAAC
C2	iPCRtagT26	TTCCTGCT	D2	iPCRtagT38	TCTCACGG
C3	iPCRtagT27	TAGTGACT	D3	iPCRtagT39	TCAGGAGG
C4	iPCRtagT28	TACAGGAT	D4	iPCRtagT40	TAAGTTCG
C5	iPCRtagT29	TCCTCAAT	D5	iPCRtagT41	TCCAGTCG
C6	iPCRtagT30	TGTGGTTG	D6	iPCRtagT42	TGTATGCG
C7	iPCRtagT31	TAGTCTTG	D7	iPCRtagT43	TCATTGAG
C8	iPCRtagT32	TTCCATTG	D8	iPCRtagT44	TGGCTCAG
C9	iPCRtagT33	TCGAAGTG	D9	iPCRtagT45	TATGCCAG
C10	iPCRtagT34	TAACGCTG	D10	iPCRtagT46	TCAGATTC
C11	iPCRtagT35	TTGGTATG	D11	iPCRtagT47	TACTAGTC
C12	iPCRtagT36	TGAACTGG	D12	iPCRtagT48	TTCAGCTC

Figure 10. ThruPLEX DNA-seq Single Indexes: Each well of the Single Index Plate contains a unique Illumina-compatible 8nt Sanger index sequence. The first 12 ThruPLEX DNA-seq single indexes share the same sequence in the first 6 bases (shown in BOLD) as the Illumina TruSeq LT indexes AD001 through AD012.

ThruPLEX DNA-seq Single-Indexed Library Structure

5' AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCTTCCGATCT-----Insert-----

-----Insert-----AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**NNNNNNNN**ATCTCGTATGCCGTCTTCTGCTTG **3'**

Sanger index

Figure 11. ThruPLEX DNA-seq single-indexed library structure: Libraries prepared from the ThruPLEX DNA-seq Kit contain the 8nt Sanger index on the 3' end.

Plate Handling Instructions for Low Throughput Applications

ThruPLEX DNA-seq 48S Kit is designed for high throughput applications; therefore, the experiment should be designed to pool and sequence the full set of 48 libraries using the entire plate of Indexing Reagents. If Indexing Reagents from the entire plate are not used at the same time, it is critical to follow the instructions below to avoid cross contamination:

- After removing Indexing Reagents of choice, cover any pierced or used index wells with scientific tape (e.g., VWR, CAT. NO. 89097-920, General-Purpose Laboratory Labeling Tape, 0.5").
- Thoroughly wipe the seal with 70% ethanol and allow it to dry completely.
- Replace the plastic lid, return the SIP to its sleeve and store at –20°C.

The Index Plate should not be frozen and thawed more than 4 times.

Multiplexing and Index Pooling

Multiplexing and pooling less than the full set of 48 libraries is possible on the MiSeq.

- **Note:** For MiSeq RTA v1.17.28 and later, base pair diversity of indexes is no longer checked by IEM because low-plexity index reads can be processed for all applications; any combination of indexes can be pooled for sequencing on MiSeq.

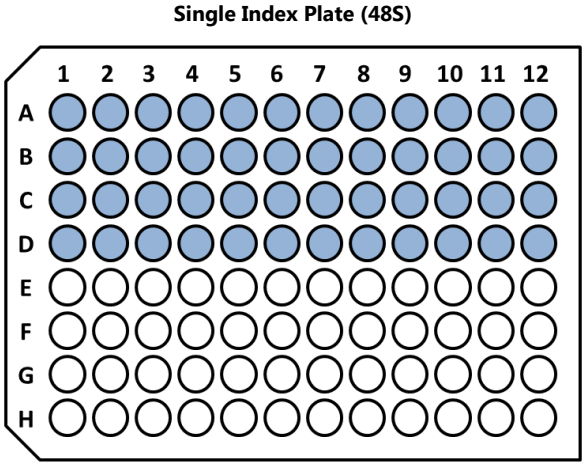

Figure 12. Single Index Plate maps with well locations: The 48S Single Index Plate contains Illumina-compatible indexes with 8nt Sanger sequences. The colored wells indicate well positions containing Indexing Reagents.

Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool that allows you to create and edit Sample Sheets for Illumina sequencers. Make sure the latest version of IEM (version 1.8 or later) is installed.

Create a Sample Sheet using the IEM, then manually copy and paste the appropriate 8nt single index sequences to be used to the CSV file of the Sample Sheet.

Index sequences can be downloaded as a CSV file at the ThruPLEX DNA-seq Product Page, under the Resources tab: http://rubicongenomics.com/products/thruplex-dna-seq-kit/.

- **Note:** The IEM will not check for color-balanced index combinations when indexes are entered manually from the CSV file.

D. ThruPLEX DNA-seq 48D and 96D Kits

Dual Index Sequences

ThruPLEX DNA-seq dual indexes are 8nt long and identical to the Illumina TruSeq HT i5 and i7 dual indexes. Each well of the Dual Index Plate (DIP) contains a unique combination of the dual index sequences (Figure 13 and Figure 15). The prepared library has the structure shown in Figure 14.

ThruPLEX DNA-seq Dual Indexes			
i7 Index	**Sequence**	**i5 Index**	**Sequence**
D701	ATTACTCG	D501	TATAGCCT
D702	TCCGGAGA	D502	ATAGAGGC
D703	CGCTCATT	D503	CCTATCCT
D704	GAGATTCC	D504	GGCTCTGA
D705	ATTCAGAA	D505	AGGCGAAG
D706	GAATTCGT	D506	TAATCTTA
D707	CTGAAGCT	D507	CAGGACGT
D708	TAATGCGC	D508	GTACTGAC
D709	CGGCTATG		
D710	TCCGCGAA		
D711	TCTCGCGC		
D712	AGCGATAG		

Figure 13. ThruPLEX DNA-seq Dual Indexes: Each well of the Dual Index Plate contains a unique combination of the 8nt Illumina TruSeq HT i7 and i5 dual index sequences.

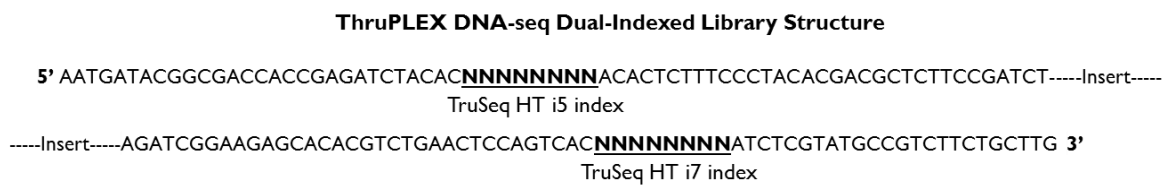

Figure 14. ThruPLEX DNA-seq dual-indexed library structure: Libraries prepared from the ThruPLEX DNA-seq Kit contain the Illumina TruSeq HT i5 index on the 5′ end and i7 index on the 3′ end.

Plate Handling Instructions for Low Throughput Applications

It is recommended that your experiment be designed to use the entire plate of Indexing Reagents to avoid contamination. However, the DIP can also be used for low level multiplexing of a small number of samples. The plate should not be frozen and thawed more than 4 times. If Indexing Reagents from

the entire plate are not used at the same time, it is it is critical to follow the instructions below to avoid cross contamination:

- After removing Indexing Reagents of choice, cover any pierced or used index wells with scientific tape (e.g., VWR, CAT. NO. 89097-920, General-Purpose Laboratory Labeling Tape, 0.5").
- Thoroughly wipe the seal with 70% ethanol and allow it to dry completely.
- Replace the plastic lid, return the DIP to its sleeve and store at –20°C.

Multiplexing and Index Pooling

It is important to select appropriate dual index combinations such that they are unique and meet the Illumina recommended compatibility requirements. In general, for pooling multiple samples, it is recommended to use indexes spanning as many columns and rows as possible to increase the diversity of the chosen combinations.

In the color-coded plate maps in Figure 15, wells with identical colors indicate one way that index combinations can be pooled together for an 8-plex experiment. For additional low-plex (2- to 16-plex) pooling guidelines, please refer to Illumina's TruSeq Sample Preparation Pooling Guide (Illumina, Document # 15042173 v01, 2015).

- **Note:** For MiSeq RTA v1.17.28 and later, base pair diversity of indexes is no longer checked by IEM because low-plexity index reads can be processed for all applications; any combination of indexes can be pooled for sequencing on MiSeq.

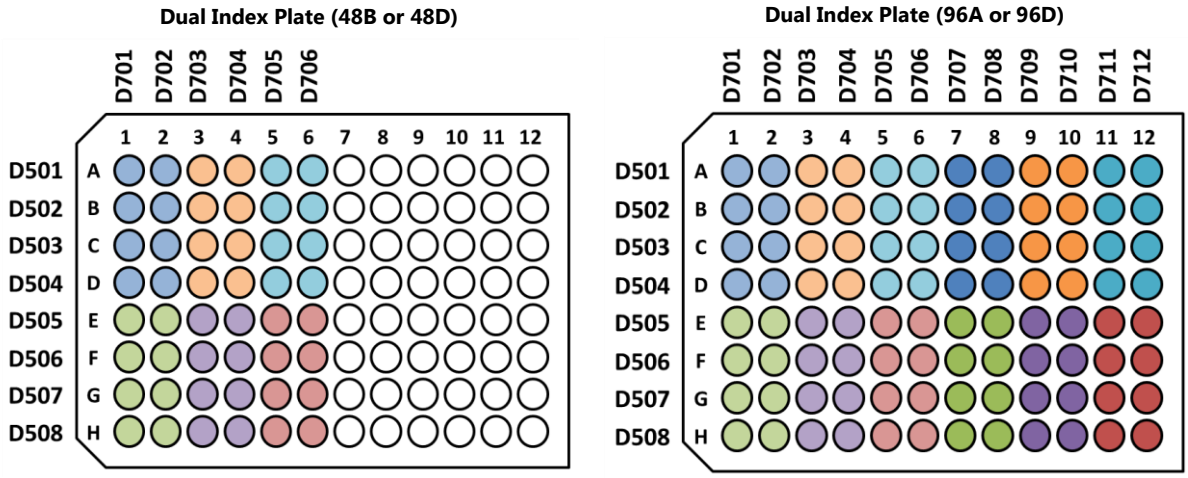

Figure 15. Dual Index Plate maps with well locations: The 96D (or 96A) and 48D (or 48B) Index Plates contain the 8nt Illumina TruSeq HT i7 and i5 dual index sequences. The dual index combination at each well position is indicated by the column (i7) and row (i5) labels on the plate maps. Wells with identical colors indicate **one** way to pool an 8-plex experiment. For additional low-plex (2- to 16-plex) pooling guideline, please refer to Illumina's TruSeq Sample Preparation Pooling Guide (Illumina, Part # 15042173 Rev B, 2014).

- **Note:** Dual Index Plates (48B) and (48D) are identical; Dual Index Plates (96A) and (96D) are also identical.

Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool that allows you to create and edit Sample Sheets for Illumina sequencers. Index combinations may be validated using the IEM, which notifies user when improper combinations are used. Make sure the latest version of IEM (version 1.8 or later) is installed.

In the IEM, on the "Workflow Parameters" page, select "TruSeq HT" in the dropdown menu for "Sample Prep Kit". Add indexes to be used on the "Sample Selection" page by clicking "Add Blank Row" and then choosing the appropriate indexes from the "index 1(I7)" and "index 2 (I5)" dropdown menus.

Appendix 2. 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 C.III.**)
NTC amplification curve appears early or produces a yield similar to sample reaction products	NTC contaminated with DNA	Use a fresh control sample and check all reagents; replace kit if necessary Clean area thoroughly and use PCR-dedicated plastics and pipettes
After purification of the amplified library, Bioanalyzer traces shows multiple peaks besides the markers	Input sample contains unevenly fragmented DNA of various sizes (e.g., plasma DNA)	If possible, quantify and check input DNA prior to using the kit. Sequencing is still recommended.
After purification of the amplified library, Bioanalyzer traces shows 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 ≤ 500 pg/µL. Repeat Bioanalyzer run.

Technical Support

For technical support contact **support@rubicongenomics.com** or call +1.734.677.4845 (9 AM – 5:30 PM EST).