



# ThruPLEX<sup>®</sup> Tag-seq Kit Instruction Manual

Illumina<sup>®</sup> NGS Library Preparation  
with Unique Molecular Tags

Research Use Only

## Product Use Limitations

ThruPLEX® Tag-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 Tag-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.

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## Product Description

ThruPLEX® Tag-seq adds unique molecular tags (UMT) to our proprietary ThruPLEX chemistry for constructing molecularly tagged and sample-indexed Illumina® NGS libraries from cell-free DNA (cfDNA) and fragmented double-stranded DNA (dsDNA). ThruPLEX stem-loop adapters now provide more than 16 million unique molecular tags to label individual fragments, reducing the false positive rate and ensuring the correct base call through data analysis. ThruPLEX Tag-seq libraries can be enriched using commercially available or custom designed capture panels from leading target enrichment platforms, such as Agilent SureSelect®, Roche NimbleGen® SeqCap® EZ, and IDT xGEN® to detect rare alleles.

## Kit Contents

ThruPLEX Tag-seq Kit contains enough buffers and enzymes to prepare master mixes for up to 4 separate uses. **Freeze thaw of reagents should be limited to no more than four times.** Contents of ThruPLEX Tag-seq Kit are not interchangeable with other Rubicon Genomics' products.

**Table 1: ThruPLEX Tag-seq Kit Contents**

Name	Cap Color	6S Kit R400584 6 Single Indexes 12 Reactions	48S Kit R400585 48 Single Indexes 48 Reactions	96D Kit R400586 96 Dual Indexes 96 Reactions
Template Preparation Buffer	Red	1 Tube	1 Tube	2 Tubes
Template Preparation Enzyme	Red	1 Tube	1 Tube	2 Tubes
Library Synthesis Buffer	Yellow	1 Tube	1 Tube	2 Tubes
Library Synthesis Enzyme	Yellow	1 Tube	1 Tube	2 Tubes
Library Amplification Buffer	Green	1 Tube	1 Tube	2 Tubes
Library Amplification Enzyme	Green	1 Tube	1 Tube	2 Tubes
Nuclease-Free Water	Clear	1 Tube	1 Tube	1 Tube
Indexing Reagents	Blue	6 Tubes	1 Single Index Plate (48S)	1 Dual Index Plate (96D)
Quick Protocol	N/A	1 Each	1 Each	1 Each

## Shipping and Storage

ThruPLEX Tag-seq Kit is shipped on dry ice. The kit must be stored at  $-20^{\circ}\text{C}$  upon arrival.

## Quality Control

ThruPLEX Tag-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 lab coat, protective goggles and disposable gloves to ensure personal safety as well as to limit potential cross contaminations during the sample preparation and subsequent amplification reactions. For more information, please refer to the appropriate Safety Data Sheets (SDS) available online at <http://rubicongenomics.com/resources/sds/>.

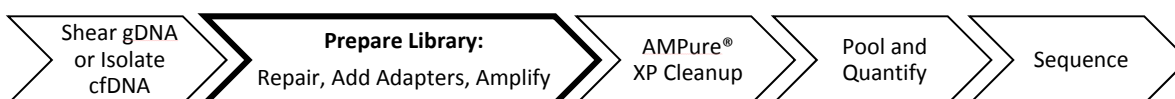
## Technical Assistance

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

## A. Introduction

Research in next generation sequencing (NGS) is rapidly evolving, and the ability to confidently detect low-frequency alleles or discriminate between molecules is now critical to the development of highly sensitive, NGS-based assays frequently used in cancer research, developmental research involving the fetal fraction derived from maternal plasma and other fields. Regardless of the application, a DNA library must first be prepared from each sample in order to be sequenced on Illumina NGS platforms. Library preparation is a critical step in the NGS workflow (Figure 1) and has a direct impact on the quality of sequencing results. The process involves placing Illumina-compatible sequencing adapters with molecular tags on DNA fragments and adding Illumina-compatible indexes for identification of individual samples. Multiple samples are then pooled (multiplexed) and sequenced in parallel.

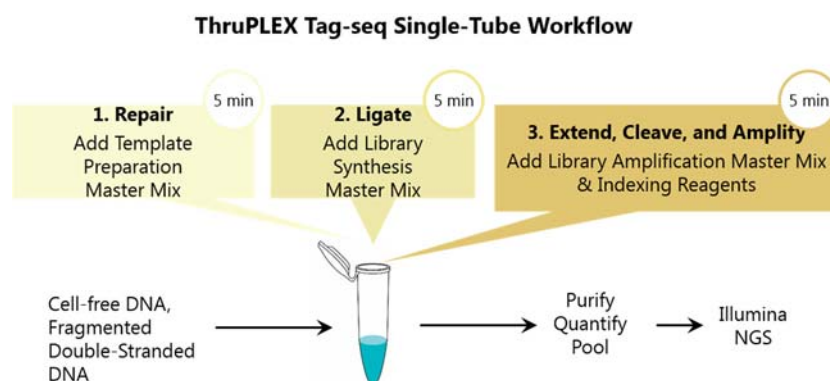
### Illumina® NGS Workflow



**Figure 1. Illumina NGS workflow:** Genomic DNA (gDNA) samples are sheared or cfDNA is isolated from plasma. Library preparation follows, consisting of repair, Illumina adapter addition, and amplification. Indexed libraries are purified, pooled, and quantified prior to sequencing on Illumina NGS platforms.

There are several key challenges when detecting low-frequency alleles and differentiating DNA fragments through sequencing. First, different sample types, such as cfDNA, have limited, highly-degraded material that can make generating high-quality libraries difficult. Furthermore, PCR artifacts can be introduced during library preparation and errors can occur during sequencing, both of which are often identified as false positives. The use of NGS samples also necessitates careful tracking. A protocol in which the sample never leaves the tube is advantageous to ensure accurate sample tracking and to avoid contamination, which would lead to false positives.

The commitment to overcome these challenges is the core of the ThruPLEX Tag-seq Kit. The ThruPLEX adapters have been redesigned to include molecular tags. Each kit contains more than 16 million unique sequences used to “tag” each DNA molecule. This allows detection of low-frequency alleles and the ability to differentiate between molecules at high sensitivity and specificity. The performance of the libraries is highly reproducible between replicates and sequencing runs and from sample to sample. Finally, the three-step, single-tube library preparation workflow (Figure 2) is the simplest in the industry, minimizing handling errors and loss of valuable samples.



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

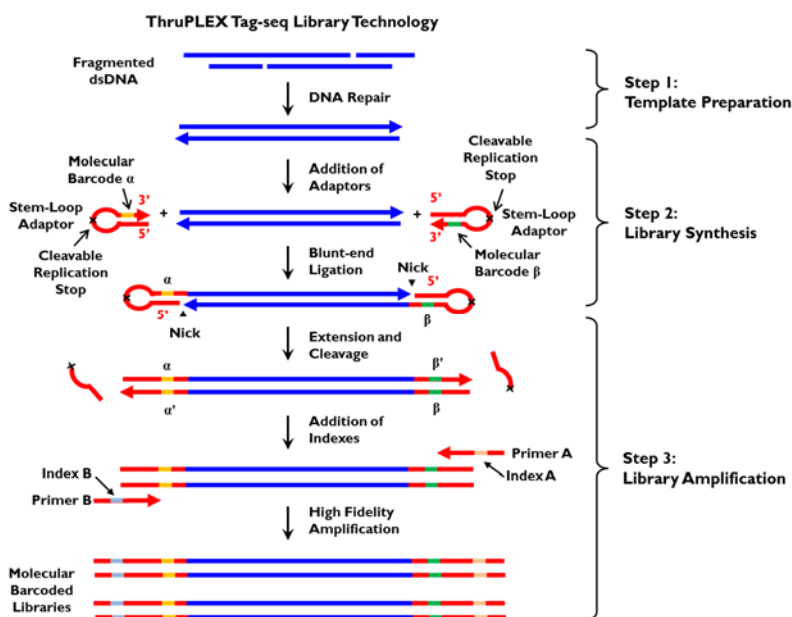
## B. ThruPLEX Tag-seq Kit

### I. Overview

ThruPLEX Tag-seq combines molecular tags with ThruPLEX chemistry to construct molecularly tagged and sample-indexed Illumina NGS libraries. Each kit contains more than 16 million unique sequences used to tag individual DNA fragments prior to amplification, providing tracking of the fragments through the library preparation, target enrichment, and the data analysis processes to detect low-frequency alleles or count individual fragments. The ThruPLEX chemistry is engineered and optimized to produce highly diverse libraries with reproducible sequencing performance from 1 to 50 ng of DNA. The entire three-step workflow takes place in a single tube or well in about 2 hours. No intermediate purification steps and no sample transfers are necessary, which prevent handling errors and loss of valuable samples. ThruPLEX Tag-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.

### II. Principle

The ThruPLEX Tag-seq Kit is based on Rubicon Genomics' patented ThruPLEX technology (Figure 3). Unlike other NGS library preparation kits, which are based on the ligation of Y-adaptors, ThruPLEX uses stem-loop adaptors with added molecular tags to construct high-quality tagged and sample-indexed libraries in a fast and efficient workflow. In the first step, Template Preparation, DNA is repaired and yields molecules with blunt ends. In the next step, stem-loop adaptors containing unique molecular tags with blocked 5' ends are ligated with high efficiency to the 5' end of the DNA, leaving a nick at the 3' end of the target fragment. Additionally, the stem-loop adaptors do not have single-strand overhangs preventing ligation to each other, both of which contribute to non-specific background found with many other NGS preparations. In the final step, the 3' ends of the DNA are extended to complete library synthesis and Illumina-compatible indexes are added through a high-fidelity amplification. Any remaining free adaptors are degraded. Hands-on time and risk of contamination are minimized by using a single-tube protocol and eliminating intermediate purifications.



**Figure 3. ThruPLEX Tag-seq Library Technology:** A three-step, single-tube reaction that starts with fragmented, dsDNA or isolated cDNA. The ThruPLEX stem-loop adaptors with molecular tags are blunt-end ligated to the repaired input DNA. These molecules are extended, then amplified using a high-fidelity polymerase to yield molecularly tagged and sample-indexed Illumina NGS library.

### III. ThruPLEX Tag-seq Workflow

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

- **Template Preparation** for efficient repair of the input DNA.
- **Library Synthesis** for ligation of Rubicon Genomics' patented stem-loop adapters with molecular tags.
- **Library Amplification** for extension of the template, cleavage of the stem-loop adaptors and amplification of the library. Illumina-compatible sample indexes are also introduced using a high-fidelity, low-bias DNA polymerase.

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

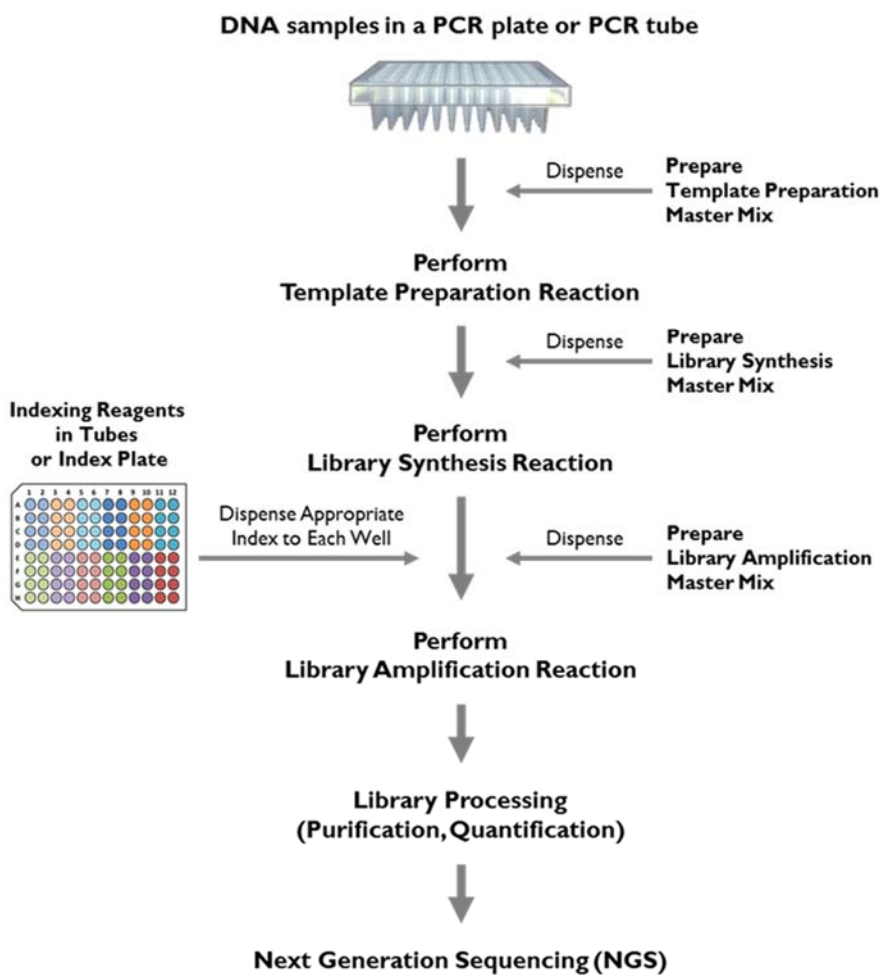


Figure 4. ThruPLEX Tag-seq library preparation workflow overview: Steps involved in ThruPLEX library preparation for Illumina NGS starting with isolated cfDNA or fragmented dsDNA.

## 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 prevent cross-contamination and 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. 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.



### III. Starting Material

DNA Sample Requirements	
Source	Plasma, cells, urine, or other biofluids, gDNA, FFPE
Type	Cell-free DNA or fragmented double-stranded DNA
Recommended Input Amount	1 ng to 50 ng
Input Volume	10 $\mu$ L
Input Buffer	$\leq$ 10 mM Tris, $\leq$ 0.1 mM EDTA

#### Blood Collection and Plasma Preparation

A reference protocol can be downloaded at the ThruPLEX Tag-seq Kit Product Page, under the Resources tab:

<http://rubicongenomics.com/products/thruplex-tag-seq-kit/>.

#### Sample Type

ThruPLEX Tag-seq Kit can be used with cfDNA and fragmented dsDNA. This kit is **not** for use with single-stranded DNA (ssDNA) or RNA.

#### DNA Isolation

**Cell-free DNA** Use a commercial cfDNA isolation kit to extract cfDNA. ThruPLEX Tag-seq Kit was optimized with cfDNA prepared using the QIAamp<sup>®</sup> Circulating Nucleic Acid Kit (Qiagen, CAT. NO. 55114).

**General Guidelines** The DNA extraction method will depend upon the particular sample type. Products and methods are designed specifically for extraction of gDNA from fresh and FFPE tissue, bacteria, etc. Regardless of the extraction method used, the DNA sample must be fragmented dsDNA in order to be used with ThruPLEX Tag-seq. This kit is **not** for use with ssDNA or RNA.

#### Input DNA Amount

The recommended input amount is 1 ng to 50 ng of DNA quantified by Qubit<sup>®</sup> Fluorometer or equivalent methods. When working with cfDNA, quantification of the mononucleosomal cfDNA fragments by Bioanalyzer<sup>®</sup> is recommended. Use an appropriate input amount of DNA to ensure sufficient variant copies are available for the library preparation process to achieve the desirable detection sensitivity. In general, detection of alleles present at lower frequencies requires higher input amount of DNA.

Estimated Genome Copies Available for Library Preparation				
Input Amount	Total Haploid Genome Copies*	Total Variant Copies at Indicated Allele Frequency		
		5%	1%	0.5%
50 ng	16,666	833	166	83
30 ng	10,000	500	100	50
10 ng	3,333	166	33	16
5 ng	1,666	83	16	8
1 ng	333	16	3	1

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

#### Input Volume

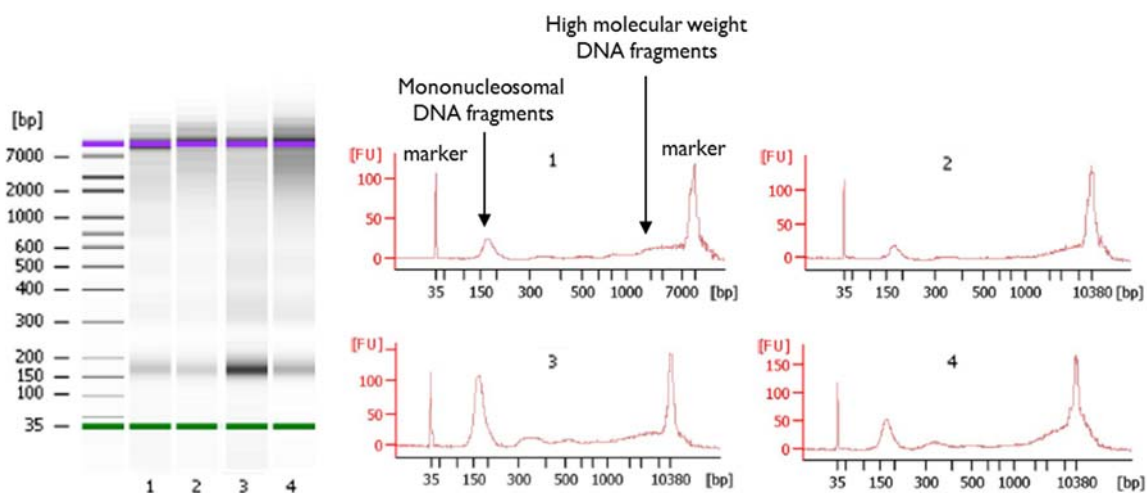
The maximum input sample volume is 10  $\mu$ L. If a sample is in a larger volume, the DNA must be concentrated to 10  $\mu$ L or less. Care should be taken to ensure the buffer concentration is appropriate (see below).

## Input Buffer

The buffer containing the input DNA must not exceed 10 mM Tris or 0.1 mM EDTA. Avoid phosphate containing buffers.

## DNA Fragment Size

**Cell-free DNA.** cfDNA contains both high and low molecular weight DNA fragments (Figure 5). The composition and concentration of the isolated cfDNA differ from sample to sample and may vary depending on the isolation method used. The cfDNA species of most interest is the mononucleosomal DNA fragments of about 170 bp in length, for which the concentration can be determined using the Agilent Bioanalyzer.



**Figure 5. Fragment size distribution of cfDNA isolated from plasma:** cfDNA from 4 different human plasma samples was isolated and analyzed using the Agilent Bioanalyzer. One distinct feature of the isolated cfDNA is a peak (or band) centered around 170 bp and a distribution of higher molecular weight fragments.

**General Guidelines.** ThruPLEX Tag-seq can be used to prepare libraries from dsDNA with fragment sizes less than 1,000 bp. ThruPLEX Tag-seq Kit is a ligation-based technology and adapters added during the process result in an approximately 170 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 (For more information, view our [Library Purification with AMPure XP beads](#) recommendations).

## IV. Positive and Negative Controls

If possible, include appropriate positive and negative controls in the experimental design to 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 the inclusion of controls will help elucidate such problems. Always prepare fresh dilutions of reference DNA. Include negative controls (No Template Control, NTC) in low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) or nuclease-free water. The positive control and experimental samples should produce indexed libraries, while the NTC should not amplify.

## V. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared just in time at each workflow step based on the number of reactions to be performed. Prepare ~5% excess of each master mix to allow for pipetting losses. Each kit contains sufficient reagents to prepare master mixes up to 4 separate times.

Transfer enzymes onto ice just prior to use and centrifuge briefly to collect contents at the bottom of the tube prior to use. Thaw the buffers at room temperature, 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 air bubbles and briefly centrifuge prior to dispensing into the PCR plate or tube(s).

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

## VI. Indexing Reagents

ThruPLEX Tag-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 from the ThruPLEX Tag-seq Kit Product Page, under the Resources tab: <http://rubicongenomics.com/products/thruplex-tag-seq-kit/>.

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

### ThruPLEX Tag-seq 6S Kit

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

### ThruPLEX Tag-seq 48S 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 pipette 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. Target Enrichment

Target enrichment is combined with ThruPLEX Tag-seq to enable deep sequencing of genomic regions of interest in a cost-effective manner. Relatively deep sequencing coverage is required to take full advantage of the unique molecular tags in ThruPLEX Tag-seq libraries. ThruPLEX Tag-seq is compatible with the major exome and target enrichment products, including [Agilent SureSelect](#), [Roche NimbleGen SeqCap EZ](#), [IDT XGen® Lockdown](#), as well as custom panels. ThruPLEX Tag-seq target enrichment protocols and application notes can be accessed through the Applications section of the Rubicon Genomics website at: <http://rubicongenomics.com/applications/enrichment/>.

## VIII. Sequencing Depth

In addition to input amount, sequencing depth is another factor that determines detection sensitivity. While sufficient variant molecules must be present in the input DNA to be detected downstream (see Section C.III.), sufficient coverage is required to utilize the unique molecular tags in ThruPLEX Tag-seq libraries to build consensus sequences. In general, detection of alleles present at lower frequencies requires sequencing to a higher depth. The prerequisite to higher depth of sequencing is a high complexity library that allows new reads to be found (non-saturation). ThruPLEX offers this quality in a consistent way for every sample.

Estimated Mean Raw Sequencing Depth Required*			
Minimum number of Unique Variant Molecules to Make a Confident Call	Allele Frequency		
	5%	1%	0.5%
3	600X	3,000X	6,000X
5	1,000X	5,000X	10,000X
10	2,000X	10,000X	20,000X

\*Raw sequencing depth includes all reads prior to removal of duplicates. This is calculated using a target peak amplification family size of 10 reads per unique molecule.

## IX. Using Illumina Experiment Manager

Make sure the latest version of the Illumina Experiment Manager (IEM) is installed (version 1.9 or later). Prior to starting the ThruPLEX Tag-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.

## X. Bioinformatic Analysis\*

To benefit from the information provided by the unique molecular tags (UMT) in ThruPLEX Tag-seq libraries, the sequencing data must be processed to group reads into amplification families and construct consensus reads. Two bioinformatics solutions designed specifically for processing data from ThruPLEX Tag-seq libraries are available as shown below. Other open-source tools can also be modified to process data from ThruPLEX Tag-seq libraries. Analysis results are dependent on the data processing algorithms used by different tools, as well as user settings for various parameters in the tools.

### Curio

Curio is a cloud-based bioinformatics platform available through Curio Genomics. Sequencing data from ThruPLEX Tag-seq libraries can be easily uploaded, processed and visualized. The platform features an ultra-fast and user-friendly interface and a powerful alignment viewer. The platform is also equipped with modules for performing variant detection, analysis and visualization. For more information, visit [www.curio-genomics.com](http://www.curio-genomics.com).

### Connor

Connor is an open-source bioinformatics tool hosted on GitHub (<https://github.com/umich-brcf-bioinf/Connor>) for processing sequencing data generated from ThruPLEX Tag-seq libraries. Connor takes an aligned BAM file as input, processes the UMT information and generates a processed BAM file containing consensus sequences. The output BAM file can be used with variant callers, such as FreeBayes, VarScan2 or GATK HaplotypeCaller. For instructions on installing and using Connor, please refer to the Data Processing Guide at the Resources section of the ThruPLEX Tag-seq product page: <http://rubicongenomics.com/products/thruplex-tag-seq-kit/> or the github page, listed above.

\*Rubicon Genomics is not responsible for these Bioinformatic solutions.

## D. ThruPLEX Tag-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  $\mu\text{L}$  of DNA into each PCR tube or well of a PCR plate.
  - **Positive control reactions using reference DNA:** If desired, include a positive control DNA.
  - **Negative control reactions/No Template Controls (NTCs):** If desired, assemble NTCs with 10  $\mu\text{L}$  of nuclease-free water or low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).
- **Note:** The maximum volume of DNA cannot exceed 10  $\mu\text{L}$ .
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	4 $\mu\text{L}$
Template Preparation Enzyme	Red	1 $\mu\text{L}$

- **Note:** Prepare 5% excess to allow for pipetting losses.
3. Assemble the **Template Preparation Reactions Mixture** as shown in the table below. To each 10  $\mu\text{L}$  sample from step 1 above, add 5  $\mu\text{L}$  of the **Template Preparation Master Mix**.

Template Preparation Reaction Mixture	
Component	Volume/Reaction
DNA Sample	10 $\mu\text{L}$
Template Preparation Master Mix	5 $\mu\text{L}$
Total Volume	15 $\mu\text{L}$

4. Mix thoroughly with a pipette. Avoid introduction of air bubbles.
5. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
6. Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each well or tube.
7. Place the plate or tube(s) in a thermal cycler with heated lid set to 101°C – 105°C. Perform the **Template Preparation Reaction** using the conditions in the table below:

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

8. After the thermal cycler reaches 22°C, remove the plate or tube(s) and centrifuge briefly.
9. Proceed to the **Library Synthesis Step**.
  - **Note:** Following the Template Preparation Step, continue to Library Synthesis Step in the same plate or tube(s).

## II. Library Synthesis Step

### Library Synthesis Reagents

Library Synthesis Reagents	
Reagent	Cap
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	2.5 µL
Library Synthesis Enzyme	Yellow	2.5 µL

- **Note:** Prepare 5% excess to allow for pipetting losses.
2. Remove plate seal or open the tube(s) containing the Template Preparation Reaction Product.
  3. Assemble the **Library Synthesis Reaction Mixture** as shown in the table below. To each well or tube, add 5 µL of the **Library Synthesis Master Mix**.

Library Synthesis Reaction Mixture	
Component	Volume/Reaction
Template Preparation Reaction Product	15 µL
Library Synthesis Master Mix	5 µL
Total Volume	20 µL

4. Mix thoroughly with a pipette. Avoid introduction of air bubbles.
5. Seal the PCR plate using an appropriate sealing film or tightly cap the tube(s).
6. Centrifuge briefly to collect the contents at the bottom of each well or tube.
7. Return the plate or tube(s) to the thermal cycler with 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
30°C	40 min
4°C	Hold for ≤30 min

8. After the thermal cycler reaches 4°C remove the plate or tube(s) and centrifuge briefly.
9. Continue to the **Library Amplification Step**.
  - **Note:** Following the Library Synthesis step, continue Library Amplification Reaction in the same plate or tube(s) maintained at 4°C.

### 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. Amplification does not begin until Stage 5.**

Illumina-compatible indexes are incorporated into the template library in Stage 4 using 4 cycles. In Stage 5, the resulting template is amplified; the number of cycles required at this stage is dependent on the amount of DNA used (See table below). 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.

#### 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 free DNA and the 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	4
30	5
10	6
5	7
1	11

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

## Library Amplification Reagents

Library Amplification Reagents	
Reagent	Cap Color
Library Amplification Buffer	Green
Library Amplification Enzyme	Green
Nuclease-Free Water	Clear
Fluorescent Dyes	Not Provided
Indexing Reagents	Tubes (Blue) or Index Plate

- Note:** It is critical to handle the Index Plate following the instructions provided to avoid cross contamination of indexes. If the entire Index Plate is not 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

- Prepare Indexing Reagents
  - Remove Indexing Reagents from freezer and thaw for ten minutes on the bench.
  - Spin Indexing Reagents briefly in a table top centrifuge to collect contents to the bottom.
  - Thoroughly wipe the Indexing Reagent Tubes or Index Plate foil seal with 70% ethanol and allow to dry.
- 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	21.5 $\mu$ L
Library Amplification Enzyme	Green	1.0 $\mu$ L
*Fluorescence Dyes (or Nuclease-Free Water)	Clear	2.5 $\mu$ L

- Note:** Prepare 5% excess to allow for pipetting losses.
  - 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 must not exceed 2.5  $\mu$ L.
    - Example: Mix 90  $\mu$ L of EvaGreen dye (20X in water, Biotium, CAT. NO. 31000-T) with 10  $\mu$ L of 1:500 dilution of Fluorescein Calibration Dye (Bio-Rad Laboratories, CAT. NO. 170-8780).  
Add 2.5  $\mu$ L of this mix 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 2.5  $\mu$ L of nuclease-free water per reaction in the Library Amplification Master Mix.
- Remove the seal on the PCR plate or open the tube(s) containing the Library Synthesis Reaction Product.
  - Add 25  $\mu$ L of the **Library Amplification Master Mix** to each well or tube.
  - Add 5  $\mu$ L of the appropriate Indexing Reagent to each well or tube:

Library Amplification Reaction Mixture	
Component	Volume/Reaction
Library Synthesis Reaction Product	20 $\mu$ L
Library Amplification Master Mix	25 $\mu$ L
Indexing Reagent	5 $\mu$ L
Total Volume	50 $\mu$ L



For ThruPLEX Tag-seq 48S and 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 pipette tip to pierce the seal above the specific Indexing Reagent on the Index Plate; discard the tip used for piercing.
  - Use a clean pipette tip to collect 5  $\mu$ L 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 air bubbles.
  7. Seal the PCR plate or tube(s) tightly and centrifuge briefly to collect the contents at 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. Amplification begins at Step 5.**

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	4 to 11 (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	4
30	5
10	6
5	7
1	11

9. Remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents at the bottom of each well.
  - **Note:** At this stage, samples can be processed for next generation sequencing (NGS) immediately or stored frozen at –20°C for up to 2 weeks. For instructions and recommendations on library pooling, purification, quantification and sequencing, please refer to **Section E**.

## E. Library Processing for Illumina Next Generation Sequencing

### I. Overview

This section contains guidelines for processing ThruPLEX Tag-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](mailto:support@rubicongenomics.com).

Libraries prepared from each sample will contain the specific indexes selected at the time of amplification. Follow the recommended workflow (solid arrows) in Figure 6 to process the libraries for Illumina NGS. Alternative workflow paths (dashed arrows) may be followed as needed. If libraries are prepared from similar samples with equivalent input amounts, 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. Recommendations on library pooling, purification, quantification and sequencing are described in the following sections.

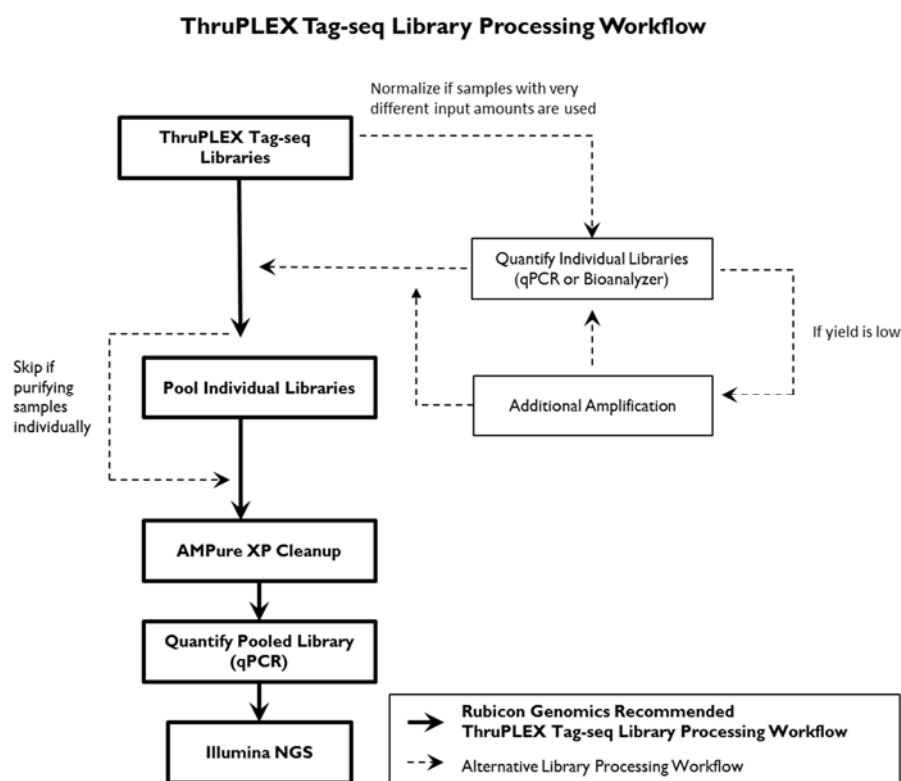


Figure 6. Workflow for processing the ThruPLEX Tag-seq amplified libraries for Illumina NGS.

### II. Library Quantification

There are several approaches available for library quantification including real-time PCR, UV absorption, fluorescence detection, and sizing and quantification using the Agilent Bioanalyzer. It is important to understand the benefits and limitations of each approach. Real-time PCR-based approaches (such as the KAPA<sup>®</sup> 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 distribution of input DNA used for library construction.

UV absorption/fluorescence detection-based methods (i.e., Nanodrop<sup>®</sup> (Thermo Scientific), Qubit Fluorometer (ThermoFisher), or Quant-iT<sup>™</sup> PicoGreen<sup>®</sup> dsDNA Assay Kit (Life Technologies)) quantify the total double-stranded nucleic acid concentration.

These methods do not discriminate adapter presence and offer no information about the size of the library molecules. The Agilent Bioanalyzer system provides sizing and quantitation information about the library analyzed, but not about the clustering competency.

### Quantification of ThruPLEX Tag-seq libraries by real-time qPCR

Use the appropriate instrument-specific KAPA Library Quantification Kit for Illumina Sequencing Platforms (Kapa Biosystems). Dilute 2 – 5  $\mu\text{L}$  of the library 100,000-fold for the quantification procedure. Since the adapters result in about 170 bp increase in the DNA fragment size, the full length of the library fragments must be used to calculate the concentration of your library. For example, for a typical cfDNA input with fragments of about 170 bp in size, use 340 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.

### Quantification of ThruPLEX Tag-seq libraries using the Bioanalyzer

Remove an aliquot of each library and dilute as appropriate in TE buffer. Load a 1  $\mu\text{L}$  aliquot of the diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, CAT. NO. 5067-4626).

## III. Additional Amplification

- **Note:** Amplification can only be performed on unpurified libraries.

If less than a desired yield of library was obtained and a plateau as measured by qPCR was not reached, the remaining library can be further amplified to attain a higher yield. ThruPLEX Tag-seq libraries can be further amplified with no extra reagents added after storage at 4°C for up to 6 hours or –20°C for up to 7 days. To perform this additional amplification, spin down the tube or plate containing the library (after thawing if the plate or tube was stored at –20°C), transfer it to a thermal cycler, and perform 2 – 3 PCR cycles as follows:

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

## IV. Library Pooling for Purification

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

Individual ThruPLEX Tag-seq libraries containing different sample indexes can be pooled at desired molar ratios to allow multiplex sequencing. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of the individual libraries, each containing a unique index. Typically, a 10  $\mu\text{L}$  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  $\mu\text{L}$ ; if 48 libraries are pooled, then the volume is 480  $\mu\text{L}$ . A 100  $\mu\text{L}$  aliquot of this pooled library is sufficient for AMPure XP purification purposes. Illumina MiSeq Control Software version 2.1 or HiSeq Control Software 2.0.12 sequencing systems require balanced index combinations to ensure proper image registration and accurate demultiplexing of the pooled samples. Please see **Appendix I** for guidelines on selecting the appropriate indexes for pooling and multiplexing or refer to Illumina instructions: [TruSeq Library Prep Pooling Guide](#) (Illumina, Part # 15042173 v01).

## V. Library Purification with AMPure XP beads

AMPure XP is the recommended method of library purification. Do not use QIAquick® cleanup or other silica-based filters for purification as this will result in incomplete removal of primers. The ratio of AMPure XP beads to library DNA is application dependent and determines the size-distribution characteristics of the library. For post-amplification purification, a 1:1 bead to sample ratio is recommended. For some samples, selection of smaller fragments is necessary and the bead to sample ratio should be increased. To collect different sized fragments, refer to the Beckman Coulter [SPRIselect User Guide](#).

### 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

### Recommended 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.
- 1. In a 1.5 mL tube, combine AMPure XP reagent with an aliquot of the pooled library at 1:1 (v/v) ratio.
- 2. Mix by pipetting 10 times to achieve a homogeneous solution; incubate for 5 min at room temperature.
- 3. Place the tube in a magnetic stand for 2 min or until the beads are captured 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 s.
    - **Note:** The volume of 80% (v/v) ethanol should be at least the volume of the sample plus AMPure XP reagent (total volume from Step 1).
  - c) Without disturbing the pellet, use a pipette to remove and discard the supernatant.
- 5. Repeat Step 4.
- 6. Incubate the tube for 2 – 5 min at room temperature with the cap open to evaporate residual ethanol. DO NOT OVER DRY THE PELLETS(S).
- 7. Remove the tube from the magnetic stand.
- 8. Resuspend the beads in an appropriate volume of 1x TE buffer (pH 8.0) or nuclease-free water.
  - **Note:** Resuspend in nuclease-free water if a vacuum concentrator will be used to concentrate the sample.
- 9. Mix by pipetting 10 times to achieve a homogeneous solution; incubate for 2 min at room temperature.
- 10. Place the tube in a magnetic stand for 1 – 2 min or until the beads are captured and the solution is clear.
- 11. With the tube in the magnetic stand and without disturbing the pellet, transfer the supernatant with a pipette into a new tube.
  - **Note:** If not used immediately, the purified library can be stored at –20 °C.

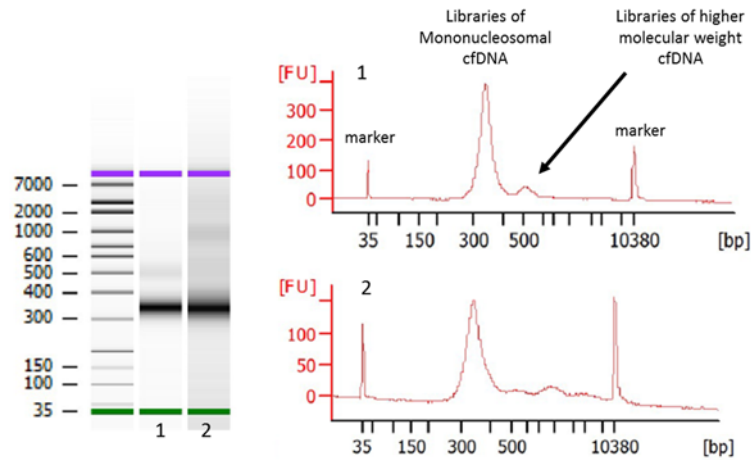
## VII. Sequencing Recommendations

ThruPLEX Tag-seq Kit generates libraries that are ready for cluster amplification and sequencing on Illumina NGS platforms using standard Illumina reagents and protocols for multiplexed libraries. Libraries prepared from cfDNA using the ThruPLEX Tag-seq

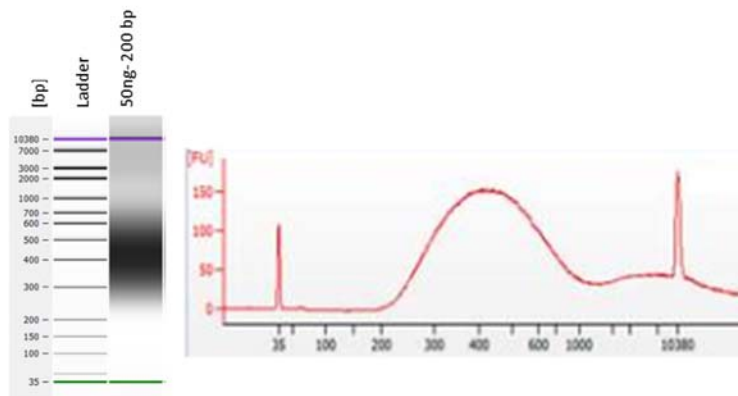
Kit result in a size distribution of library fragments centered at about 340 bp when analyzed using the Agilent Bioanalyzer (Figure 7). Libraries prepared using fragmented dsDNA result in a size distribution of library fragments that is dependent on the input DNA fragment size. For example, using a 200 bp DNA fragment size would result in a 370 bp library (Figure 8). 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 Tag-seq libraries.

Illumina recommends adding 1% PhiX control for most libraries. PhiX is a small genome that provides a balanced and diverse library to prevent sequencing problem and that can be quickly aligned to calculate error rates. For low diversity libraries and if experiencing sequencing issues, increase the PhiX control spike-in to at least 5%.

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 cfDNA libraries prepared using ThruPLEX Tag-seq:** cfDNA from plasma was isolated using the QIAamp Circulating Nucleic Acid Kit. Libraries were prepared using ThruPLEX Tag-seq Kit and analyzed using the Agilent Bioanalyzer after library amplification. One distinct feature of the amplified libraries is a dominant peak (or intense band) around 340 bp containing library products of the mononucleosomal DNA fragments and a distribution of higher molecular weight library products.



**Figure 8. Bioanalyzer analysis of fragmented dsDNA libraries prepared using ThruPLEX Tag-seq:** Libraries were prepared from 50 ng DNA (200 bp) using the ThruPLEX Tag-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 with a 1:1 bead to library would remove fragments around and below 100 bp.

# Appendix I. Indexing Reagents

## A. Overview

ThruPLEX Tag-seq Kits contain all necessary reagents to generate molecularly tagged and sample-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.

- Indexing Reagents provided with ThruPLEX Tag-seq Kit cannot be substituted with indexing reagents from any other sources.

**Table 3: ThruPLEX Tag-seq Kit – Indexing Reagents**

	<b>6S Kit</b>	<b>48S Kit</b>	<b>96D Kit</b>
<b>Number of Reactions</b>	12	48	96
<b>Number of Indexes</b>	6	48	96
<b>Index Type</b>	Single	Single	Dual
<b>Length of Indexes</b>	8nt	8nt	8nt
<b>Format</b>	6 Tubes	96-Well Plate	96-Well Plate
<b>Number of Uses</b>	Up to 4	Single	Single
<b>Illumina Experiment Manager or BaseSpace Kit Selection</b>	TruSeq LT or Manual Input	Manual Input only	TruSeq HT

If libraries will be pooled for sequencing, use the Illumina Experiment Manager (IEM) or BaseSpace to record information about your samples **before** beginning the ThruPLEX Tag-seq Library Preparation Protocol. Appendices 1B to 1D provide index sequences, plate handling instructions, multiplexing and index pooling guidelines, and IEM Sample Sheet setup instructions specific for each ThruPLEX Tag-seq Kit.

- For ThruPLEX Tag-seq 6S Kit, proceed to Appendix 1B
- For ThruPLEX Tag-seq 48S Kit, proceed to Appendix 1C
- For ThruPLEX Tag-seq 96D Kit, proceed to Appendix 1D

## B. ThruPLEX Tag-seq 6S Kits

### Single Index Sequences

ThruPLEX Tag-seq single indexes (6S) 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 first 6 bases of each 6S indexes are identical to the Illumina TruSeq LT indexes AD001 through AD006 (Figure 9), and they can be sequenced the same way as the TruSeq LT indexes. The prepared library has the structure shown in Figure 10.

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

ThruPLEX Tag-seq Single Indexes (6S)				
Tube	Sanger Tag	Sequence	TruSeq LT Index	TruSeq LT Sequence
1	iPCRtagT1	<b>ATCACGTT</b>	AD001	<b>ATCACG</b>
2	iPCRtagT2	<b>CGATGTTT</b>	AD002	<b>CGATGT</b>
3	iPCRtagT3	<b>TTAGGCAT</b>	AD003	<b>TTAGGC</b>
4	iPCRtagT4	<b>TGACCACT</b>	AD004	<b>TGACCA</b>
5	iPCRtagT5	<b>ACAGTGGT</b>	AD005	<b>ACAGTG</b>
6	iPCRtagT6	<b>GCCAATGT</b>	AD006	<b>GCCAAT</b>

**Figure 9. ThruPLEX Tag-seq Single Indexes:** Each Indexing Reagent Tube contains a unique Illumina-compatible 8nt Sanger index sequence. The first 6 bases of each 6S indexes are identical to the Illumina TruSeq LT indexes AD001 through AD006.



**Figure 10. ThruPLEX Tag-seq single-indexed library structure:** Libraries prepared from the ThruPLEX Tag-seq 6S Kit contain two 6 nucleotide UMT and two 8-11 nucleotide stems (sequences shown) on each end of the insert. The 8nt Sanger index is found on the 3' end.

### Multiplexing and Index Pooling

Color balanced index combinations are only important for MiSeq control software version 2.1 or earlier and the HiSeq control software 2.0.12 or earlier. For pooling guidelines and for Illumina sequencing systems that require balanced index combinations, refer to Illumina's [TruSeq Library Prep Pooling Guide](#) (Illumina, Part # 15042173 v01, 2015).

### Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool that creates and edits Sample Sheets for Illumina sequencers. To use this tool with ThruPLEX indexes, ensure that the latest version of IEM (version 1.9 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, only the first 6 bases of the 8nt sequence will be sequenced.

Option 2: Create a Sample Sheet using the IEM. Open the CSV file of the Sample Sheet in Microsoft Excel, then manually copy and paste the appropriate 8nt single index sequences to be used to the Sample Sheet.

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



## C. ThruPLEX Tag-seq 48S Kits

### Single Index Sequences

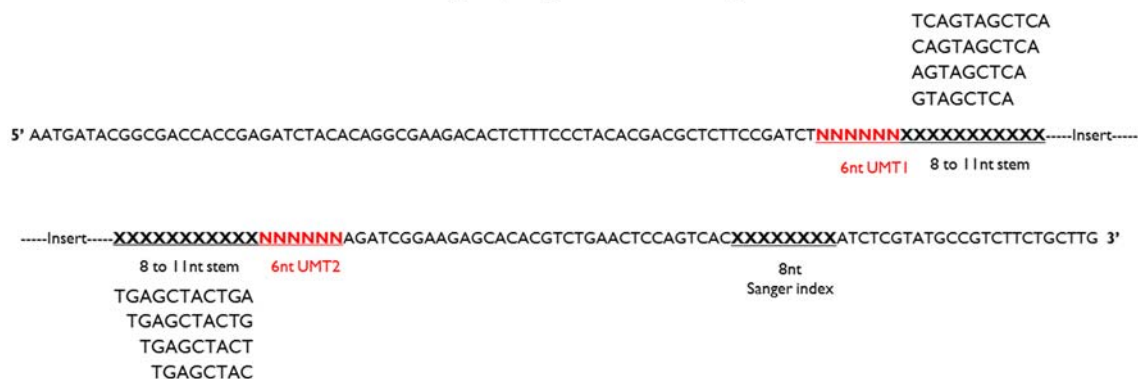
ThruPLEX Tag-seq single indexes use Illumina-compatible 8nt sequences developed by the Wellcome Trust Sanger Institute in Cambridge, UK. Each well of the Single Index Plate (SIP) contains a unique single index sequence (Figure 11). The first 12 ThruPLEX Tag-seq single indexes (wells A1 through A12) have sequences identical to the first 6 bases of the Illumina TruSeq LT indexes AD001 through AD012. The prepared library has the structure shown in Figure 12.

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

ThruPLEX Tag-seq Single Indexes					
Well	Sanger Tag	Sequence	Well	Sanger Tag	Sequence
A1	iPCRtagT1	<b>ATCACG</b> TT	B1	iPCRtagT13	TGGTTGTT
A2	iPCRtagT2	<b>CGATGT</b> TT	B2	iPCRtagT14	TCTCGGTT
A3	iPCRtagT3	<b>TTAGGC</b> AT	B3	iPCRtagT15	TAAGCGTT
A4	iPCRtagT4	<b>TGACCA</b> CT	B4	iPCRtagT16	TCCGTCTT
A5	iPCRtagT5	<b>ACAGTG</b> GT	B5	iPCRtagT17	TGTACCTT
A6	iPCRtagT6	<b>GCCAAT</b> GT	B6	iPCRtagT18	TTCTGTGT
A7	iPCRtagT7	<b>CAGATC</b> TG	B7	iPCRtagT19	TCTGCTGT
A8	iPCRtagT8	<b>ACTTGAT</b> G	B8	iPCRtagT20	TTGGAGGT
A9	iPCRtagT9	<b>GATCAG</b> CG	B9	iPCRtagT21	TCGAGCGT
A10	iPCRtagT10	<b>TAGCTT</b> GT	B10	iPCRtagT22	TGATACGT
A11	iPCRtagT11	<b>GGCTAC</b> AG	B11	iPCRtagT99	GTGCTACC
A12	iPCRtagT12	<b>CTTGTA</b> 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 11. ThruPLEX Tag-seq Single Indexes:** Each well of the Single Index Plate contains a unique Illumina-compatible 8nt Sanger index sequence. The first 12 ThruPLEX Tag-seq single indexes have identical sequences to the first 6 bases (shown in BOLD) as the Illumina TruSeq LT indexes AD001 through AD012.

## ThruPLEX Tag-seq Single-Indexed Library Structure



**Figure 12: ThruPLEX Tag-seq single-indexed library structure:** Libraries prepared from the ThruPLEX Tag-seq 48S Kit contain two 6 nucleotide UMT and two 8-11 nucleotide stems (sequences shown) on each end of the insert. The 8nt Sanger index is found on the 3' end.

### Plate Handling Instructions for Low Throughput Applications

It is recommended that experiments be designed to use the entire plate of Indexing Reagents. However, the SIP 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 critical to follow the instructions below to avoid cross contamination:

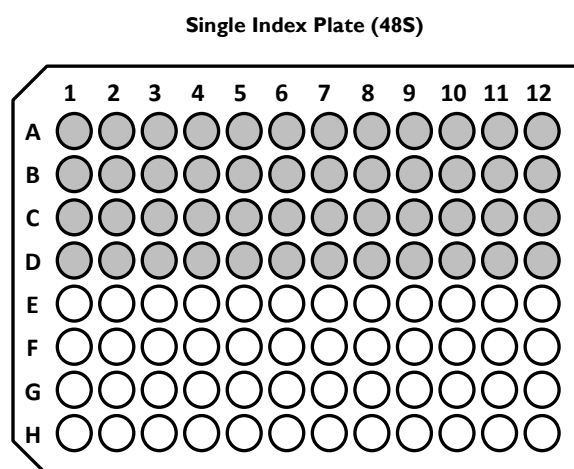
- After removing Indexing Reagents of choice, cover any pierced or used index wells with scientific tape (e.g., VWR, CAT. NO. 89097-920, General-Purpose Laboratory Labeling Tape, 0.5”).
- Thoroughly wipe the seal with 70% ethanol and allow it to dry completely.
- Replace the plastic lid, return the SIP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

### Multiplexing and Index Pooling

For Illumina sequencing systems that require balanced index combinations (Miseq Control Software 2.1 or earlier and HiSeq Control Software 2.0.12 or earlier), select appropriate single indexes that are unique and meet Illumina recommended compatibility requirements (Figure 13 and Figure 14). For more information, refer to Illumina's [TruSeq Library Prep Pooling Guide](#) (Illumina, Part # 15042173 v01, 2015).

		Well	Sanger Tag	Sequence
3-plex	Option A	A1	iPCRtagT1	ATCACGTT
		A7	iPCRtagT7	CAGATCTG
		B12	iPCRtagT101	GGTTGGAC
	Option B	A2	iPCRtagT2	CGATGTTT
		A8	iPCRtagT8	ACTTGATG
		D1	iPCRtagT102	GGCACAAC
	Option C	A5	iPCRtagT5	ACAGTGGT
		B11	iPCRtagT99	GTGCTACC
		D10	iPCRtagT46	TCAGATTC
	Option D	A12	iPCRtagT12	CTTGTA CT
		D11	iPCRtagT47	TACTAGTC
		D12	iPCRtagT48	TTCAGCTC
4-plex or greater	Use 3-plex options with any other available indexes			

**Figure 13. Low-plex pooling guidelines for ThruPLEX Tag-seq 48S indexes:** For pooling 3 or more samples, use the 3 required indexes specified in one of the options above plus any other available index as needed.



**Figure 14. Single Index Plate map with well locations:** The 48S Single Index Plate contains Illumina-compatible indexes with 8nt Sanger sequences. The shaded wells indicate well positions containing Indexing Reagents.

### Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool to create and edit Sample Sheets for Illumina sequencers. Make sure the latest version of IEM (version 1.9 or later) is installed.

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

Index sequences can be downloaded as a CSV file at the ThruPLEX Tag-seq Product Page, under the Resources tab:

<http://rubicongenomics.com/products/thruplex-tag-seq-kit/>.

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

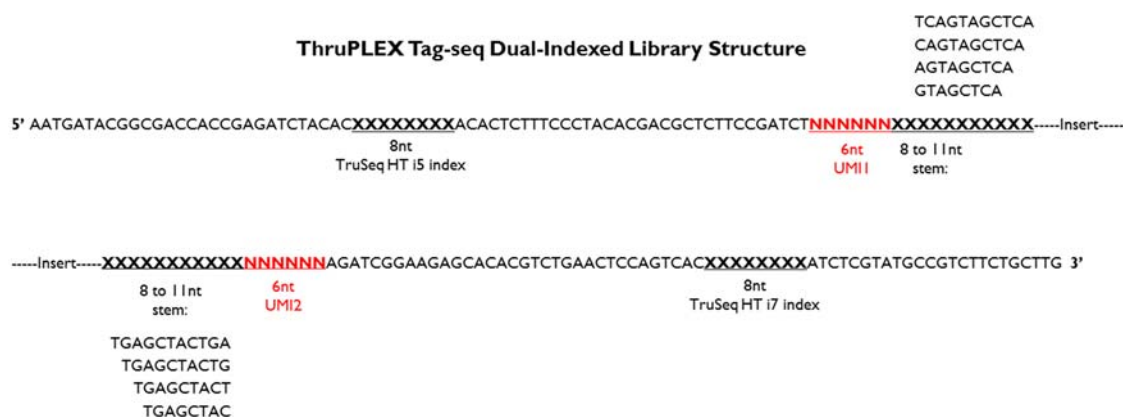
## D. ThruPLEX Tag-seq 96D Kits

### Dual Index Sequences

ThruPLEX Tag-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 15 and Figure 16).

ThruPLEX Tag-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 15. ThruPLEX Tag-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 16. ThruPLEX Tag-seq Dual-Indexed Library Structure:** Libraries prepared from the ThruPLEX Tag-seq 96D Kit contain two 6 nucleotide UMT and two 8-11 nucleotide stems (sequences shown) on each end of the insert. The Illumina TruSeq HT i5 index is found on the 5' end and i7 index on the 3' end.

### Plate Handling Instructions for Low-Throughput Applications

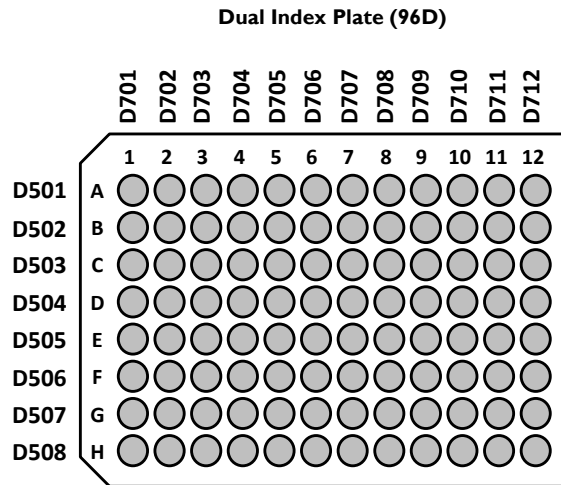
It is recommended that experiments be designed to use the entire plate of Indexing Reagents. However, the DIP can also be used for low-level multiplexing of a small number of samples. The plate should not be frozen and thawed more than 4 times. If Indexing Reagents from the entire plate are not used at the same time, it is 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  $-20^{\circ}\text{C}$ .

## Multiplexing and Index Pooling

For Illumina sequencing systems that require balanced index combinations (Miseq Control Software 2.1 or earlier and HiSeq Control Software 2.0.12 or earlier), select appropriate dual index combinations that are unique and meet 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. For low-plex (2- to 16-plex) pooling guidelines and for Illumina sequencing systems that require balanced index combinations, refer to Illumina's [TruSeq Library Prep Pooling Guide](#) (Illumina, Part # 15042173 v01, 2015).



**Figure 17. Dual Index Plate map with well locations:** The 96D Index Plate contains 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 map.

## Sample Sheet Setup

The Illumina Experiment Manager (IEM) is a desktop tool 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 1.9 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 ( <b>Section C.III.</b> )
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 show broad peak(s) extending from less than 1,000 bp to greater than 1,000 bp	Library over-amplified or Bioanalyzer chip overloaded (common for high sensitivity chips)	Perform fewer PCR cycles at Stage 5 of the Library Amplification Reaction. For high sensitivity chips, load $\leq 500$ pg/ $\mu$ L. Repeat Bioanalyzer run.

## Technical Support

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

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