ThruPLEX® Tag-seq Kit Quick Protocol

ThruPLEX® Tag-seq combines molecular tags with our proprietary ThruPLEX chemistry, allowing construction of molecularly tagged and sample-indexed Illumina® NGS libraries. Each kit contains all necessary reagents for preparing indexed Illumina NGS libraries, including Illumina-compatible adapters and indexing reagents. Each kit provides sufficient reagents for manual use up to 4 separate times. For more information, please visit http://rubicongenomics.com/products/thruplex-tag-seq-kit/.

For a detailed protocol, **refer to the ThruPLEX Tag-seq Kit Instruction Manual** at www.rubicongenomics.com/resources/manuals/.

Storage: Store kit at –20 °C upon arrival.

Technical support: Call (734) 677-4845 (9AM-5:30PM Eastern Time) or contact support@rubicongenomics.com.

Kit Contents

Name	Cap Color	6S Kit CAT. NO. R00584	48S Kit CAT. NO. R00585	96D Kit CAT. NO. R00586
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		6 Tubes	1 Single Index Plate	1 Dual Index Plate
Quick Protocol				

Input DNA Sample Requirements

	Requirement
Source	DNA from plasma, cells, urine, other biofluids, gDNA
Type	Cell-free DNA; fragmented double-stranded DNA
DNA Fragment Size	< 1000 bp
Recommended Input Amount*	1 – 50 ng
Input volume	10 µL
Input buffer	≤ 10 mM Tris, ≤ 0.1 mM EDTA

*Quantified by Qubit® Fluorometer or equivalent methods. Quantification of the mononucleosomal cfDNA fragments by Bioanalyzer® is recommended.

A. Notes Before Starting

1. **Input DNA Sample Requirements (Table above, right):** Please refer to the ThruPLEX Tag-seq Kit Instruction Manual for detailed instructions on preparing DNA samples.
2. **Additional Materials and Equipment Needed:** Thermal cycler with 50 µL reaction volume capability and heated lid; centrifuge; PCR tubes or plates; PCR plate seals; low-binding barrier tips; fluorescent dyes; Agencourt® AMPure® XP (Beckman Coulter, CAT. NO. A63880), 80% v/v Ethanol.
3. **PCR Plates/Tubes:** Select plates/tubes that are compatible with the thermal cyclers and/or real-time PCR instruments used. Ensure that there is no evaporation during the cycling process by using proper seal/caps as **evaporation may reduce reproducibility**.
4. **Positive and Negative Controls:** If desired, include a positive control DNA and a No Template Control (NTC) as a negative control in parallel to ensure that the reaction proceeded as expected.
5. **Preparation of Master Mixes:** 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. **Keep all enzymes, buffers, and master mixes on ice until use.**
   - 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.
   - 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 used.
6. **Indexing Reagents:** Indexing Reagents can be frozen and thawed no more than four times.
   - The 6S Kit is provided with 6 Indexing Reagents pre-dispensed in tubes. They have sufficient reagents for up to 4 uses and contain 8-nucleotide Sanger indexes that share the same sequences in the first 6 bases as the Illumina TruSeq® LT indexes AD001 through AD006.
   - The 48S Kit is provided with a Single Index Plate (SIP) containing 48 Illumina-compatible single indexes, each with a unique 8-nucleotide Sanger index sequence. Each well has sufficient volume for a single use.
   - The 96D Kit is provided with a Dual Index Plate (DIP) containing 96 Illumina-compatible dual indexes. Each well has sufficient volume for a single use and contains a unique combination of Illumina's 8-nucleotide TruSeq HT i5 and i7 index sequences.
7. **Index Plate Handling Instructions:** The Index Plate is sealed with pierceable sealing foil. Follow the instructions below to avoid cross contamination.
   - Thaw the Index Plate for 10 min on the bench top prior to use. Once thawed, briefly centrifuge the plate to collect the contents at the bottom of each well. Thoroughly wipe the foil seal with 70% ethanol and allow it to dry completely.
   - Pierce the seal above each well containing the specific index combination with a clean 20 µL pipette tip with filter barrier; discard the tip.
   - Use a new pipette tip to collect 5 µL of a specific index combination and add it to the reaction mixture at the Library Amplification Step. A multichannel pipette may be used if needed. If indexes from the entire plate are not used at the same time, follow the instructions below to avoid contamination:
     - Cover any pierced index wells with scientific tape (such as VWR General Scientific Tape 0.5", CAT. NO. 89097-920) to mark the index as used.
     - Wipe the seal with 70% ethanol and let it dry completely. Replace the plastic lid, return the plate to its sleeve and store at –20°C.
8. **Low Level Multiplexing:** Select appropriate index combinations that meet Illumina recommended compatibility requirements. For more information on multiplexing and index pooling, refer to the ThruPLEX Tag-seq Kit Instruction Manual at http://rubicongenomics.com/resources/manuals/.
9. **Index Sequences and Index Plate Maps:** Refer to the ThruPLEX Tag-seq Index Guide at http://rubicongenomics.com/resources/manuals/.
10. **Library Purification, Quantification, and Sequencing:** For instructions and recommendations, refer to the ThruPLEX Tag-seq Instruction Manual at http://rubicongenomics.com/resources/manuals/.

ThruPLEX® Tag-seq Kit is 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. ThruPLEX Tag-seq is 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.

B. Quick Protocol

I. Template Preparation Step

1. Add 10 µL of DNA sample to each well of a PCR plate or tube. If desired, include control samples.
2. Depending on the number of reactions, prepare the **Template Preparation Master Mix** as described in the table below. Mix thoroughly with a pipette. Keep on ice until used.

Template Preparation Master Mix		
Component	**Cap Color**	**Volume/Rxn**
Template Preparation Buffer	Red	4 µL
Template Preparation Enzyme	Red	1 µL

3. To each 10 µL sample from step 1 above, add **5 µL** of the **Template Preparation Master Mix**.
4. Mix thoroughly with a pipette.

■ **Note:** Final volume at this stage will be 15 µL.

5. Seal the PCR plate using proper sealing film or tightly cap the tube(s).
6. Centrifuge briefly to collect contents to the bottom of each well or tube.
7. Place the plate or tube(s) in a thermal cycler with a heated lid set to 101–105°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 ≤ 2 hours

8. Remove the plate or tube(s) from the thermal cycler and centrifuge briefly.
9. Continue to the Library Synthesis Step.

II. Library Synthesis Step

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

Library Synthesis Master Mix		
Component	**Cap Color**	**Volume/Rxn**
Library Synthesis Buffer	Yellow	2.5 µL
Library Synthesis Enzyme	Yellow	2.5 µL

2. Remove the seal on the plate or open the tube(s).
3. Add **5 µL** of the **Library Synthesis Master Mix** to each well or tube.
4. Mix thoroughly with a pipette.

■ **Note:** Final volume at this stage is 20 µL.

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

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

8. Remove the plate or tube(s) from the thermal cycler and centrifuge briefly.
9. Continue to the Library Amplification Step.

III. Library Amplification Step

1. Remove the Indexing Reagents from the freezer and thaw for 10 min on bench top. Prior to use, centrifuge the Indexing Reagents to collect the contents at the bottom. Wipe the Index Plate foil seal with 70% ethanol and allow to dry.
2. Prepare **Library Amplification Master Mix** as described in the table below. Mix thoroughly with a pipette. Keep on ice until used.

Library Amplification Master Mix		
Component	**Cap Color**	**Volume/Rxn**
Library Amplification Buffer	Green	21.5 µL
Library Amplification Enzyme	Green	1.0 µL
Fluorescence Dyes (or Nuclease-Free Water)		2.5 µL

■ **Fluorescence Dyes** for detection and optical calibration are added when monitoring amplification in real time during cycling. Refer to the real-time PCR instrument's user manual for calibration dye recommendations. The volume of dyes should not exceed 2.5 µL. If a regular thermal cycler is used, there is no need to add the dyes; replace with 2.5 µL of nuclease-free water.

■ **Example: EvaGreen®/Fluorescein dye mix.** Prepare by mixing 9:1 v/v ratio of EvaGreen Dye (20X in water, Biotium, CAT. NO. 31000-T) and 1:500 diluted Fluorescein Calibration Dye (Bio-Rad Laboratories, CAT. NO. 170-8780); add 2.5 µL of this mix per reaction.

3. Remove the seal on the PCR plate or open the tube(s).
4. Add **25 µL** of **Library Amplification Master Mix** to each well or tube.
5. Add **5 µL** of the appropriate **Indexing Reagent** to each well or tube.

■ **Note:** For the 48S and 96D Kits, follow the Index Plate handling instructions (section A.7) to avoid index cross contamination.

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

■ **Note:** Final volume at this stage is 50 µL.

7. Seal the plate or tube(s) tightly and centrifuge briefly to collect contents to the bottom of each well or tube.
8. Return plate or tube(s) to the real time PCR thermal cycler/thermal cycler with a heated lid set to 101–105°C. Perform **Library Amplification Reaction** using the cycling conditions from 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**	**No. 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 to11 see table below
		*72°C	50 s	
	6	4°C	Hold	1
*Acquire fluorescence data at this step, if monitoring in real-time.				

■ **Selecting the optimal number of amplification cycles:** The first 4 stages are **NOT** amplification steps. The number of PCR cycles required at Stage 5 of the Library Amplification Reaction is dependent upon the amount of input DNA and the thermal cycler used. The table below provides the suggested number of PCR cycles at Stage 5 for different input amounts.

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 this protocol, the typical yields range from 500 ng to 1000 ng.

9. Remove the plate or tube(s) from the thermal cycler and centrifuge briefly.

■ **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 the ThruPLEX Tag-seq Kit Instruction Manual at http://rubicongenomics.com/resources/manuals/.

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