ThruPLEX® Tag-seq Kit Protocol-At-A-Glance

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 four separate times. For more information, visit http://rubicongenomics.com/products/thruplex-tag-seq-kit/. For a detailed protocol, refer to the ThruPLEX Tag-seq Kit User Manual.

Storage and Handling

Store the kit at –20˚C upon arrival. Prior to use, transfer enzymes to ice and centrifuge briefly. Thaw buffers, vortex briefly, and centrifuge prior to use. Keep all enzymes and buffers on ice until used.

Kit Contents

Name	Cap Color	6S (12 Rxn) Cat. No. R00584	48S Cat. No. R00585	96D Cat. No. R00586
Template Preparation Buffer	Red	1 Tube	1 Tube	2 Tube
Template Preparation Enzyme	Red	1 Tube	1 Tube	2 Tube
Library Synthesis Buffer	Yellow	1 Tube	1 Tube	2 Tube
Library Synthesis Enzyme	Yellow	1 Tube	1 Tube	2 Tube
Library Amplification Buffer	Green	1 Tube	1 Tube	2 Tube
Library Amplification Enzyme	Green	1 Tube	1 Tube	2 Tube
Nuclease-Free Water	Clear	1 Tube	1 Tube	1 Tube
Indexing Reagents		6 Tubes	1 Single Index Plate	1 Dual Index Plate

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	<1,000 bp
Recommended Input Amount*	1–50 ng
Input Volume	50 pg–50 ng
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.

NOTE: Please refer to the ThruPLEX Tag-seq Kit User Manual for detailed instructions on preparing DNA samples.

Notes Before Starting

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 (for bead purification), and 70% v/v ethanol.

Selecting PCR plates/tubes: Select plates/tubes that area compatible with the thermal cyclers and/or real-time PCR instruments used. Ensure that there is no evaporation during the process by using proper seals/caps during cycling as *evaporation may reduce reproducibility*.

Positive and negative controls: If necessary, include a positive control DNA (e.g., Coriell DNA, Covaris sheared, 200–300 bp) and a no-template control (NTC) as a negative control in parallel to ensure that the reaction proceeded as expected.

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 four separate times. Keep all enzymes and buffers on ice.

- 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 min of the previous step's cycling protocol and kept on ice until used.

Indexing Reagents: Indexing Reagents can be frozen and thawed no more than four times.

- The 6S Kit is provided with six Indexing Reagents pre-dispensed in tubes. They have sufficient reagents for up to four uses and contain 8-nucleotide Sanger indexes that share the same sequences in the first six bases as the lllumina TruSeq® LT indexes AD001 through AD006.
- The 48S Kit is provided with a Single Index Plate (SIP) containing 48 lllumina-compatible single indexes, each with a unique 8-nt 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 lllumina-compatible dual indexes. Each well has sufficient volume for a single use and contains a unique combination of lllumina's 8-nt TruSeq HT i5 and i7 index sequences.

Index Plate handling instructions: Follow the instructions given below to avoid index cross-contamination.

- Thaw the Index Plate for 10 min on the benchtop prior to use. Once thawed, briefly centrifuge the plate to collect the contents to 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 200-µl filtered pipet tip; discard the tip.
- Use a new pipet 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 tape (e.g., VWR General Scientific Tape 0.5", Cat. No. 89097-920) to mark the index as used.
  - Once the Index Plate is used, wipe the seal with 70% ethanol and let it dry completely. Replace the plastic lid and return the plate to its sleeve. Store at –20˚C.

Low-level multiplexing: Select index combinations that meet the Illumina-recommended compatibility requirements. For more information on multiplexing and index pooling, please refer to Appendix A of the ThruPLEX Tag-seq Kit User Manual.

Index sequences and Index Plate maps: Please refer to Appendix A of the ThruPLEX Tag-seq Kit User Manual.

Library purification, quantification, and sequencing: Please refer to Section IV.B. Library Processing for Illumina Next Generation Sequencing of the ThruPLEX Tag-seq Kit User Manual.

Template Preparation

1. Add 10 µl of DNA sample to each well of a PCR plate or tube. If necessary, 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/Reaction
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 at the bottom of each well or tube.
7. Place the plate or tube(s) in a thermal cycler with a heated lid set to 101°C–105°C. Perform the Template Preparation Reaction using the conditions in the table below.

Template Preparation Reaction

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

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

Library Synthesis

1. Prepare the 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/Reaction
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 the Library Synthesis Reaction using the conditions in the next table.

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 Library Amplification.

Library Amplification

1. Remove the Indexing Reagents from the freezer and thaw for 10 min on the bench top. Prior to use, centrifuge the Indexing Reagents to collect the contents at the bottom. For the 48S kit, wipe the SIP foil seal with 70% ethanol and allow to dry.
2. Prepare the 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
Nuclease-Free Water (plus fluorescent dyes; see NOTES)		2.5 µl

NOTES:

- Fluorescent dyes (for detection and optical calibration) are added when monitoring amplification in real time during cycling. Please refer to the real-time PCR Instrument's user manual for calibration dye recommendations. The volume of detection and calibration dyes plus Nuclease-Free Water should not exceed 2.5 µl. If a regular thermal cycler is used, there is no need to add the dyes; use 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 (on Page 1) 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 at the bottom of each well or tube.
8. Return the plate or tube(s) to the real-time PCR thermal cycler/thermal cycler with a heated lid set to 101°C–105°C. Perform the 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	# 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 67°C 72°C*	20 sec 20 sec 40 sec	4
Library Amplification	5	98°C 72°C	20 sec 50 sec	5–11**
	6	4°C	Hold	1

*** If monitoring in real-time, acquire fluorescence data here.**
****See NOTE and table below, Stage 5 Amplification Guide.**

NOTE: The first four 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)	# of Cycles
50	4
30	5
10	6
5	7
1	11

NOTE: 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 to 1,000 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° for up to two weeks. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to the ThruPLEX Tag-seq Kit User Manual at http://rubicongenomics.com/resources/manuals/.

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