ThruPLEX® DNA-seq Kit Single Index Protocol-At-A-Glance

ThruPLEX DNA-seq builds on the innovative ThruPLEX chemistry to generate DNA libraries with expanded multiplexing capability and with even greater performance. Kits contain either 6, 12, or 24 single-read 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. This protocol is for use with the following kits: ThruPLEX DNA-seq 6S (12) Kit (Cat. No. R400523), ThruPLEX DNA-seq 12S Kit (Cat. No. R400429), ThruPLEX DNA-seq 12S Kit (48 Rxn) (Cat. No. R400428), and ThruPLEX DNA-seq 48S Kit (Cat. No. R400427). For more information, visit http://rubicongenomics.com/products/thruplex-dna-seq-kit/. For a detailed protocol, refer to the ThruPLEX DNA-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)	12S	12S (48 Rxn)	48S
Template Preparation Buffer	Red	1 Tube	1 Tube	1 Tube	1 Tube
Template Preparation Enzyme	Red	1 Tube	1 Tube	1 Tube	1 Tube
Library Synthesis Buffer	Yellow	1 Tube	1 Tube	1 Tube	1 Tube
Library Synthesis Enzyme	Yellow	1 Tube	1 Tube	1 Tube	1 Tube
Library Amplification Buffer	Green	1 Tube	1 Tube	1 Tube	1 Tube
Library Amplification Enzyme	Green	1 Tube	1 Tube	1 Tube	1 Tube
Nuclease-Free Water	Clear	1 Tube	1 Tube	1 Tube	1 Tube
Indexing Reagents	Blue	6 Tubes	12 Tubes	12 Tubes	1 Single Index Plate

Input DNA Sample Requirements

	Requirement
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–50 ng
Input Volume	10 µl
Input Buffer	≤10 mM Tris, ≤0.1 mM EDTA

NOTE: Please refer to the ThruPLEX DNA-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: Keep all enzymes and buffers on ice. 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.

- ThruPLEX DNA-seq 48 S Kit is provided with a Single Index Plate (SIP) containing 48 Ilumina-compatible indexes, each with a unique 8-nt Sanger index sequence (see table below). The SIP is sealed with pierceable sealing foil; each well has sufficient volume for a single use.
- ThruPLEX DNA-seq 12S and 12S (48 Rxn) Kits are provided with 12 Indexing Reagents pre-dispensed in tubes. They have sufficient reagents for up to eight uses and contain 8-nt Sanger indexes (see table below) that share the same sequences in the first six bases as the Illumina TruSeq® LT indexes AD001 through AD012.
- ThruPLEX DNA-seq 6S (12 Rxn) Kit is provided with 6 Indexing Reagents (Tubes 1–6 in the table below), pre-dispensed in tubes. They have sufficient reagents for up to eight uses and contain 8-nt Sanger indexes that share the same sequences in the first six bases as the Illumina TruSeq LT indexes AD001 through AD006.

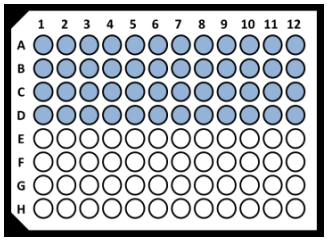
Single Index Plate (SIP) handling instructions: Follow the instructions given below to avoid index cross-contamination.

- Thaw the SIP for 10 min on the benchtop 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 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 (MiSeq® system only, see "low-level multiplexing"), 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 SIP 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 the ThruPLEX DNA-seq 48S Kit, multiplexing less than the full set of 48 libraries is possible on the MiSeq system only because MiSeq RTA v1.17.28 and later can process low-plexity index reads. For more information on multiplexing and index pooling, please refer to Appendix 1 of the ThruPLEX DNA-seq Kit User Manual.

Index Plate Maps:

Single Index Plate (48S)



The colored wells indicate those that contain Indexing Reagents.

ThruPLEX DNA-seq Single Indexes

Well	Sequence	Well	Sequence	Well	Sequence	Well	Sequence
A1	ATCACGTT	B1	TGGTTGTT	C1	TGCGATCT	D1	GGCACAAC
A2	CGATGTTT	B2	TCTCGGTT	C2	TTCCTGCT	D2	TCTCACGG
A3	TTAGGCAT	B3	TAAGCGTT	C3	TAGTGACT	D3	TCAGGAGG
A4	TGACCACT	B4	TCCGTCTT	C4	TACAGGAT	D4	TAAGTTCG
A5	ACAGTGGT	B5	TGTACCTT	C5	TCCTCAAT	D5	TCCAGTCG
A6	GCCAATGT	B6	TTCTGTGT	C6	TGTGGTTG	D6	TGTATGCG
A7	CAGATCTG	B7	TCTGCTGT	C7	TAGTCTTG	D7	TCATTGAG
A8	ACTTGATG	B8	TTGGAGGT	C8	TTCCATTG	D8	TGGCTCAG
A9	GATCAGCG	B9	TCGAGCGT	C9	TCGAAGTG	D9	TATGCCAG
A10	TAGCTTGT	B10	TGATACGT	C10	TAACGCTG	D10	TCAGATTC
A11	GGCTACAG	B11	GTGCTACC	C11	TTGGTATG	D11	TACTAGTC
A12	CTTGTACT	B12	GGTTGGAC	C12	TGAACTGG	D12	TTCAGCTC

Tube	Sequence
1	ATCACGTT
2	CGATGTTT
3	TTAGGCAT
4	TGACCACT
5	ACAGTGGT
6	GCCAATGT
7	CAGATCTG
8	ACTTGATG
9	GATCAGCG
10	TAGCTTGT
11	GGCTACAG
12	CTTGTACT

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Template Preparation

1. Add 10 µl of DNA sample to each well of a PCR plate or tube. If necessary, include NTC negative control buffer sample(s) and positive 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	2 µl
Template Preparation Enzyme	Red	1 µl

3. To each 10-µl sample from Step 1 above, add 3 µl of the Template Preparation Master Mix.
4. Mix thoroughly with a pipette.

NOTE: Final volume at this stage will be 13 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	1 µl
Library Synthesis Enzyme	Yellow	1 µl

2. Remove the seal on the plate or open the tube(s).
3. Add 2 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 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. 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
22°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/ Reaction
Library Amplification Buffer	Green	25 µl
Library Amplification Enzyme	Green	1 µl
Nuclease-Free Water (plus fluorescent dyes; see NOTES)	Clear	4 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 4 µl. If a regular thermal cycler is used, there is no need to add the dyes; use 4 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 and 1.5 µl of Nuclease-Free Water per reaction.

3. Remove the seal on the PCR plate or open the tube(s).
4. Add 30 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 kit, follow the SIP 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	# 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	5–16**
	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 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. We recommend performing an optimization experiment to identify the appropriate number of PCR cycles needed. 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	DNA Input (ng)	# of Cycles
50	5	2	10
20	6	1	11
10	7	0.2	14
5	8	0.05	16

NOTES:

- The amount of amplified library can range from 100 ng to 1 µg, depending on sample type, fragmentation size, and thermal cycler used. When using Covaris-fragmented reference DNA with an average size of 200 bp and following the recommended number of amplification cycles, the typical yield is 300–700 ng.
- Over-amplification could result in a higher rate of PCR duplicates in the library.

9. Remove samples 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° and processed later. For instructions on library pooling, purification, quantification, and sequencing, see the ThruPLEX DNA-seq Kit User Manual: http://rubicongenomics.com/resources/manuals/.

This document has been reviewed and approved by the Quality Department.

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