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

ThruPLEX DNA-seq builds on the innovative ThruPLEX chemistry to generate DNA libraries with expanded multiplexing capability and 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 48D Kit (48 Rxn) (Cat. No. R400406) and ThruPLEX DNA-seq 96D Kit (Cat. No. R400407). 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
Template Preparation Buffer	Red
Template Preparation Enzyme	Red
Library Synthesis Buffer	Yellow
Library Synthesis Enzyme	Yellow
Library Amplification Buffer	Green
Library Amplification Enzyme	Green
Nuclease-Free Water	Clear
Indexing Reagents	1 Dual 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

ThruPLEX DNA-seq Dual Indexes

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

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 are 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: ThruPLEX DNA-seq is designed for high-throughput applications. It is provided with a Dual Index Plate (DIP) containing either 48 or 96 Illumina-compatible dual indexes. Each well of the DIP has sufficient volume of Indexing Reagent for a single use and contains a unique combination of Illumina's 8-nucleotide TruSeq® HT i5 and i7 index sequences (see table, above right).

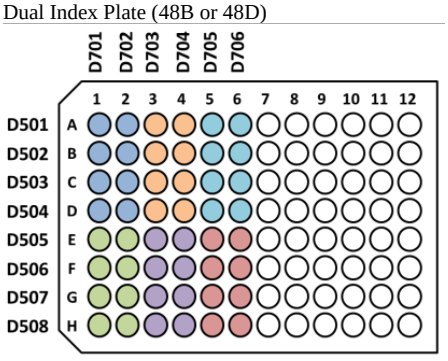
Dual Index Plate (DIP) handling instructions: The DIP is sealed with pierceable sealing foil and can be frozen and thawed no more than four times. Follow the instructions below to avoid index cross-contamination.

- Thaw the DIP 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 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.

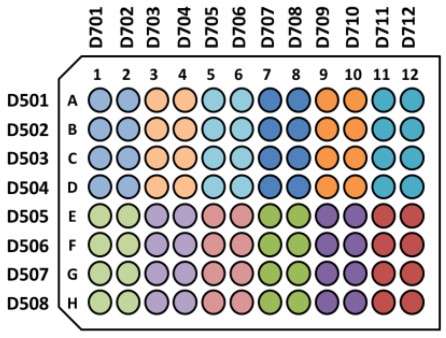
Low-level multiplexing: Select index combinations that meet the Illumina-recommended compatibility requirements. For more information on multiplexing and index pooling, please see the plate maps below and refer to Appendix A of the ThruPLEX DNA-seq Kit User Manual.

Index Plate maps:

Dual Index Plate (48B or 48D)



Dual Index Plate (96A or 96D)



The index combination at each well position is indicated by the column (i7) and row (i5) labels on the plate maps. The well colors illustrate one way to pool dual-index combinations for an 8-plex experiment; wells sharing the same color should be pooled together. For other ways to pool a low-plex (2- to 16-plex) experiment, please refer to Illumina's TruSeq Sample Preparation Pooling Guide (Illumina, Part No. 15042173 Rev B, 2014).

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 DIP from the freezer and thaw for 10 min on the bench top. Prior to use, centrifuge the DIP to collect the contents at the bottom. 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: Follow the DIP 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 following tables.

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
50	5
20	6
10	7
5	8
2	10
1	11
0.2	14
0.05	16

NOTE: Over-amplification could result in a higher rate of PCR duplicates in the library.

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° and processed later. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to the ThruPLEX DNA-seq Kit User Manual at http://rubicongenomics.com/resources/manuals/.

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

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada 800.662.2566
Asia Pacific +1.650.919.7300
Europe +33.(0)1.3904.6880
Japan +81.(0)77.565.6999