ThruPLEX® DNA-seq Kit Quick Protocol, Dual Indexes

For Use With: ThruPLEX DNA-seq 48D Kit, CAT. NO. R400406
ThruPLEX DNA-seq 96D Kit, CAT. NO. R400407

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 48 or 96 dual read Illumina®-compatible indexes pre-dispensed and sealed in microplates. 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. For more information, please visit www.rubicongenomics.com/products/ThruPLEX-DNA-seq/.

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

Storage and Handling: Store 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.**

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

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
Quick Protocol	

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	< 1000 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		

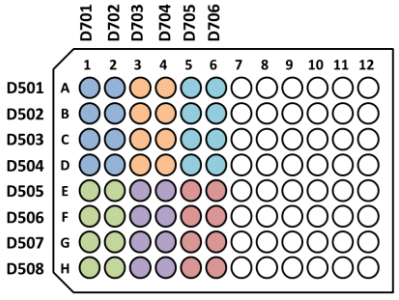
A. Kit Contents

- See table above left.

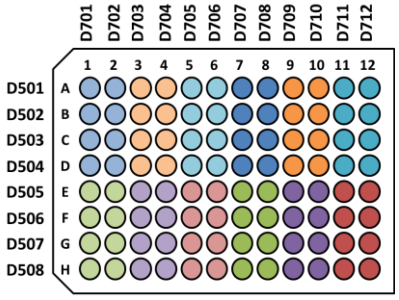
B. Notes before starting

1. **Input DNA sample requirements:**
   - See table above middle. Please refer to the ThruPLEX DNA-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. **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 seal/caps during cycling as **evaporation may reduce reproducibility**.
4. **Positive and Negative Controls:** If necessary, include a positive control DNA (eg. 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.
5. **Preparation of Master Mixes:** Keep all enzymes and buffers on ice. 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:** 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).
7. **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 given below to avoid potential index cross contamination.
   - Thaw the DIP for 10 min on the bench top 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 (low level multiplexing), 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.
     - 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 and store at -20°C.
8. **Low level multiplexing:** Select appropriate dual index combinations that meet Illumina recommended compatibility requirements. For more information on multiplexing and index pooling, please see plate maps below and refer to the ThruPLEX DNA-seq Kit Instruction Manual at www.rubicongenomics.com/resources/manuals/.
9. **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# 15042173 Rev B, 2014).

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C. Quick Protocol

I. Template Preparation Step

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 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°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 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/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 **Library Synthesis Reaction** using the conditions in the table below.

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 the Library Amplification Step.

III. Library Amplification Step

1. Remove the DIP from the freezer and thaw for 10 min on bench top. Prior to use, centrifuge the DIP to collect the contents at the bottom. Wipe 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/Reaction**
Library Amplification Buffer	Green	25.0 µL
Library Amplification Enzyme	Green	1.0 µL
Nuclease Free Water (plus fluorescent dyes*)	Clear	4.0 µL

■ ***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 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** from the DIP to each well or tube.

■ **Note**: Follow the DIP handling instructions (section B.7 of this quick protocol) 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°C – 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**	**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	5 to16 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 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)	**Number 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°C and processed later. For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to the ThruPLEX DNA-seq Kit Instruction Manual at www.rubicongenomics.com/resources/manuals/.