

ThruPLEX® DNA-seq Kit Quick Protocol, Single Indexes

For Use With: ThruPLEX DNA-seq 6S Kit (12 Rxn), CAT. NO. R400523
 ThruPLEX DNA-seq 12S Kit, CAT. NO. R400429
 ThruPLEX DNA-seq 12S Kit (48 Rxn), CAT. NO. R400428
 ThruPLEX DNA-seq 48S Kit, CAT. NO. R400427

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 48 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. For more information, please visit <http://rubicongenomics.com/products/thruplex-dna-seq-kit/>.

For detailed protocol, please refer to the **ThruPLEX DNA-seq Kit Instruction Manual** at <http://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 | 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 |
| 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 |

A. Kit Contents

- See table above left.

B. Notes before starting

1. Input DNA sample requirements:

- See table above right. 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 that **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:

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

- ThruPLEX DNA-seq 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 (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 8 uses and contain 8-nucleotide Sanger indexes (see table below) that share the same sequences in the first 6 bases as the Illumina TruSeq LT indexes AD001 through AD012.
- ThruPLEX DNA-seq 6S (12 Rxn) Kit is provided with 6 Indexing Reagents (Tube 1-6 in table below) pre-dispensed in tubes. They have sufficient reagents for up to 8 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.

7. Single Index Plate (SIP) Handling Instructions:

Follow the instructions given below to avoid index cross contamination.

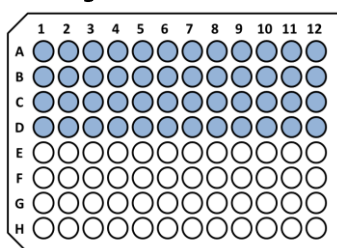
- Thaw the SIP for 10 min on the bench top prior to use. Once thawed, briefly centrifuge the plate to collect the contents to the bottom of each well. Thoroughly wipe the foil seal with 70% ethanol and allow it to dry completely.
- Pierce the seal above each well containing the specific index combination 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 (MiSeq only, see section B.8 below), 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 index combinations that meet the Illumina recommended compatibility requirements. For ThruPLEX DNA-seq 48S Kit, multiplexing less than the full set of 48 libraries is possible on MiSeq 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 Instruction Manual at <http://rubicongenomics.com/resources/manuals/>.

9. Index Plate Maps:

Single Index Plate (48S)



The colored wells indicate well positions containing Indexing Reagents.

ThruPLEX DNA-seq Single Indexes

| Well | Sequence | Well | Sequence | Well | Sequence | Well | Sequence |
|------|-----------|------|----------|------|-----------|------|-----------|
| A1 | ATCACGTT | B1 | TGGTGTGT | C1 | TGCGATCT | D1 | GGCACAAAC |
| A2 | CGATGTTT | B2 | TCTCGGTT | C2 | TTCTGCT | D2 | TCTCACGG |
| A3 | TTAGGCAT | B3 | TAAGCGTT | C3 | TAGTGA CT | 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 | TCATPGAG |
| 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 | CTTGTA CT | B12 | GGTTGGAC | C12 | TGA ACTGG | 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 | CTTGTA CT |

C. Quick Protocol

I. Template Preparation Step

- 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.
- 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 |

- To each 10 µL sample from step 1 above, add **3 µL** of the **Template Preparation Master Mix**.
- Mix thoroughly with a pipette.
- Note:** Final volume at this stage will be 13 µL.
- Seal the PCR plate using proper sealing film or tightly cap the tube(s).
- Centrifuge briefly to collect contents to the bottom of each well or tube.
- 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 |

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

II. Library Synthesis Step

- 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 |

- Remove the seal on the plate or open the tube(s).
- Add **2 µL** of the **Library Synthesis Master Mix** to each well or tube.
- Mix thoroughly with a pipette.
- Note:** Final volume at this stage is 15 µL.
- Seal the PCR plate using proper sealing film or tightly cap the tube(s).
- Centrifuge briefly to collect contents to the bottom of each well or tube.

- 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 |

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

III. Library Amplification Step

- 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. For 48S Kit, wipe SIP foil seal with 70% ethanol and allow to dry.
- 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.
- Remove the seal on the PCR plate or open the tube(s).
 - Add **30 µL** of **Library Amplification Master Mix** to each well or tube.
 - Add **5 µL** of the appropriate **Indexing Reagent** to each well or tube.
 - Note:** For 48S Kit, follow the SIP handling instructions (section B.7 of this quick protocol) to avoid index cross contamination.
 - Mix thoroughly with a pipette. Avoid introducing excessive air bubbles.
 - Note:** Final volume at this stage is 50 µL.
 - Seal the plate or tube(s) tightly and centrifuge briefly to collect contents to the bottom of each well or tube.
 - 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 to 16 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.
- 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 <http://rubicongenomics.com/resources/manuals/>.

For technical support contact support@rubicongenomics.com or call +1.734.677.4845 (9 AM – 5:30 PM EST).

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