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

ThruPLEX Plasma-seq is built with ThruPLEX chemistry to generate high-quality DNA libraries from cell-free DNA (cfDNA) extracted from plasma samples. Each kit contains all necessary reagents for preparing indexed lllumina® NGS libraries, including optimized lllumina-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-plasma-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.

Kit Contents

Name	Cap Color	12S Kit Cat. No. R400490	48S Kit Cat. No. R400491	96D Kit Cat. No. R400492
Template Preparation Buffer	Red	1 Tube	1 Tube	2 Tubes
Template Preparation Enzyme	Red	1 Tube	1 Tube	2 Tubes
Library Synthesis Buffer	Yellow	1 Tube	1 Tube	2 Tubes
Library Synthesis Enzyme	Yellow	1 Tube	1 Tube	2 Tubes
Library Amplification Buffer	Green	1 Tube	1 Tube	2 Tubes
Library Amplification Enzyme	Green	1 Tube	1 Tube	2 Tubes
Nuclease-Free Water	Clear	1 Tube	1 Tube	1 Tube
Indexing Reagents		12 Tubes	1 Single Index Plate	1 Dual Index Plate

Input DNA Sample Requirements

	Requirement
Source	Plasma
Type	Cell-free DNA
Input Amount*	1–30 ng
Input Volume	10 µl
Input Buffer	≤10 mM Tris, ≤0.1 mM EDTA

*Quantified by Qubit Fluorometer or equivalent methods.

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

Notes Before Starting

Input DNA sample requirements: See table above, right. The QIAamp Circulating Nucleic Acid Kit (Qiagen, Cat. No. 55114) is the recommended method for extracting cell-free DNA from plasma samples.

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. A63880J, 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, buffers, and master mixes on ice until use.

- 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 12S Kit is provided with 12 Indexing Reagents 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 lllumina TruSeq®·LT indexes AD001 through AD012.
- 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-nucleotide TruSeq HT i5 and i7 index sequences.

Index Plate 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 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 20 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, refer to the ThruPLEX Plasma-seq Kit Instruction Manual at http://rubicongenomics.com/resources/manuals/.

Index sequences and Index Plate maps: Refer to the ThruPLEX Plasma-seq Index Guide at http://rubicongenomics.com/resources/manuals/.

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/Rxn
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
22°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. 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 per reaction
Library Amplification Buffer	Green	21.5 µl
Library Amplification Enzyme	Green	1.0 µl
Fluorescent Dyes (or Nuclease-Free Water		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 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 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 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	**5–11****
	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 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
30	5
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 cell-free DNA and following this protocol, the typical yields range from 500 ng 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 (and processed later). For instructions and recommendations on library pooling, purification, quantification, and sequencing, please refer to the ThruPLEX Plasma-seq Kit User Manual at http://rubicongenomics.com/resources/manuals/.

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