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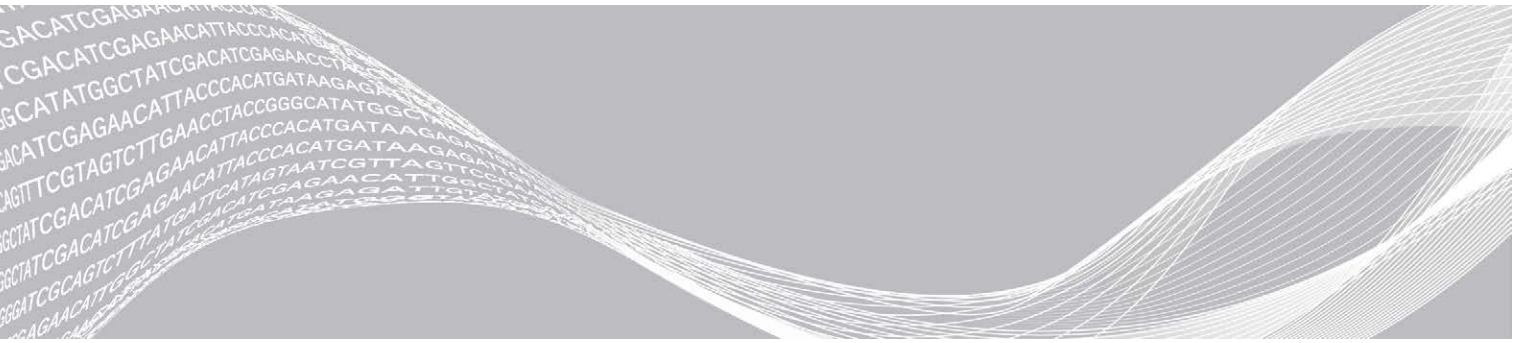
TruSight Oncology 500

Reference Guide



Document # 1000000067621 v04

ILLUMINA PROPRIETARY

November 2019

Revision History

Document	Date	Description of Change
Document # 1000000067621 v04	November 2019	Noted that guidelines regarding the number of libraries and possible DNA/RNA combinations per sequencing run are in the *NextSeq System Denature and Dilute Libraries Guide (document # 15048776)*.
Document # 1000000067621 v03	October 2019	Added an RNA only workflow. Added gene amplifications as one of the biomarkers detected by the assay. Removed RNA maximum input guidance. As a result, removed dilution concentration recommendations for RNA in the protocol. Noted that a minimum of 40 ng of DNA/RNA input is required with the kit, and that inputs lower than 40 ng can decrease library yield and quality. Removed sample concentration amount in TEB when preparing for fragmenting gDNA because more than 40 ng of DNA can be used. Added kits with PierianDx to the kit contents list. Added Covaris consumables and equipment. Added Covaris ME220 equipment settings. Added new tips and techniques for avoiding cross-contamination and sealing the plate. Noted that guidelines regarding the number of libraries and possible DNA/RNA combinations per sequencing run are on TruSight Oncology 500 support pages. Added additional mixing instructions for LNB1 bead pellets to ensure proper resuspension. Added a step in the bind sections to vortex for 1 minute to resuspend the beads, and removed that instruction from introduction and preparation information. Added information on an AccuClear quantitation kit effective for quantifying TruSight Oncology 500 libraries. Added additional guidelines for quantifying libraries, including using RSB as blank, running diluted Accuclear DNA standard and blank in triplicate, and running libraries in single replicates.
Document # 1000000067621 v02	June 2019	Updated the list of acronyms.
Document # 1000000067621 v01	March 2019	Added steps to the protocol for sample libraries derived from RNA. Added kit contents, consumables, and equipment for RNA.
Document # 1000000067621 v00	December 2018	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

The TruSight™ Oncology 500 protocol describes an enrichment-based approach to convert DNA and RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue samples into libraries enriched for cancer-related genes that can be sequenced on Illumina® sequencing systems. The TruSight Oncology 500 Kit enables the preparation of 48 libraries from DNA, RNA, or a combination of DNA and RNA libraries.

The kit is optimized to provide high sensitivity and specificity for low-frequency somatic variants across 523 genes. DNA biomarkers include the following:

- Single nucleotide variants (SNVs)
- Insertions
- Deletions
- Gene amplifications
- Multinucleotide variants (MNVs)

TruSight Oncology 500 also detects immunotherapy biomarkers for tumor mutational burden (TMB) and microsatellite instability (MSI) in DNA. Fusions and splice variants are detected in RNA.

DNA/RNA Input Recommendations

Use a minimum of 40 ng of DNA/RNA input with the TruSight Oncology 500 Kit assay. Inputs lower than 40 ng can decrease library yield and quality. Quantify the input nucleic acids before beginning the protocol. To obtain sufficient nucleic acid material, isolate nucleic acid from a minimum of 2 mm^3 of FFPE tissue.

- Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The QIAGEN AllPrep DNA/RNA FFPE Kit provides a high yield of nucleic acids.
- Use a fluorometric quantification method that uses DNA/RNA binding dyes such as AccuClear™ (DNA) or QuantiFluor® (RNA).

Assess Sample Quality

For optimal performance, assess DNA and RNA sample quality before using the TruSight Oncology 500 Kit assay.

- DNA samples can be assessed using the Illumina FFPE QC Kit.
- Use DNA samples that result in a delta Cq value ≤ 5. Samples with a delta Cq > 5 may decrease assay performance.
- RNA samples can be assessed using Advanced Analytical Technologies Fragment Analyzer™ (Standard Sensitivity RNA Analysis Kit) or Agilent Technologies 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).
- Use RNA samples that result in a DV_{200} value of ≥ 20%. Using samples with a DV_{200} value < 20% may decrease assay performance.

Reference Samples (Optional)

- Use reference materials with known variant composition when running the library preparation, such as HorizonDx HD753 (DNA) and Agilent Universal Human Reference RNA. The Agilent Universal Human Reference RNA is an intact RNA sample. Process the sample after the intact RNA procedure in *Denature and Anneal RNA* on page 12.
- Use RNase/DNase-free water as a no template control.
- Processing a reference sample or no template control reduces the total number of test samples that can be processed.

DNA Shearing Recommendations

The TruSight Oncology 500 Kit assay is optimized to prepare libraries from gDNA that are fragmented to 90–250 bp. The assay is optimized using the Covaris E220*evolution*™, LE220, or ME220 Focused-ultrasonicator with the parameters provided in *Fragment gDNA* on page 16. Fragment size distribution can vary due to differences in sample quality and the sonication instrumentation used for fragmentation.

Use the following guidelines for shearing.

- Excessive bubbles or an air gap in the shearing tube can lead to incomplete shearing.
  - Load the gDNA into the Covaris tube slowly to avoid creating bubbles.
  - Centrifuge the Covaris tube to collect the sample at the bottom of the tube before shearing.
- If you use the LE220 Covaris instrument, fill unused Covaris 8 microTUBE Strip wells with 52 µl water for optimal performance.
- [Optional] Assess fragment size distribution of sheared samples using the Agilent DNA 1000 Kit with the Agilent Bioanalyzer 2100.

Additional Resources

The TruSight Oncology 500 support pages on the Illumina website provide software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSight Oncology 500 Checklist (document # 1000000067619)	Provides a checklist of steps for the experienced user.
TruSight Oncology 500 Consumables & Equipment List (document # 1000000067617)	Provides an interactive checklist of user-provided consumables and equipment.

Chapter 2 Protocol

Introduction

This section describes the TruSight Oncology 500 Kit protocol.

- Review the complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment. For more information, please see *Kit Contents* on page 36.
- Follow the protocol in the order described using the specified parameters.
- See the *NextSeq System Denature and Dilute Libraries Guide (document # 15048776)* for guidelines on the number of libraries and possible DNA/RNA combinations per sequencing run.
- Before beginning library preparation, record sample concentration and sample quality information. Save this information for later use during data analysis.

RNA and DNA libraries may be prepared simultaneously. Illumina recommends performing the TruSight Oncology 500 Kit assay workflow according to the following schedule:

- Day 1: cDNA Synthesis from RNA samples, DNA Shearing of gDNA samples, Library Preparation, and begin Overnight (First) Hybridization. See the *Library Prep DNA Only Workflow* on page 6 and the *Library Prep DNA and RNA Workflow* on page 8.
- Day 2: Enrichment, Optional Enriched Library QC Check (Quantify Libraries), Bead-Based Normalization of Enriched Libraries, and Loading of Libraries onto Sequencing Platform. See the *Enrichment DNA Only Workflow* on page 7 and the *Enrichment DNA and RNA Workflow* on page 9.

Safe stopping points indicated throughout the protocol enable alternatives to the preceding schedule.

Tips and Techniques

Review tips and techniques before starting the protocol. Many critical techniques are listed only here, and are not repeated in the protocol.

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- Use a unidirectional workflow when moving from pre-amp to post-amp areas.
- To prevent amplification product or probe carryover, avoid returning to the pre-amp area after beginning work in the post-amp area.
- Clean work surfaces and equipment thoroughly before and after the procedure with an RNase/DNase-inhibiting cleaner.
- When adding or transferring samples, change tips between **each well**.
- Handle and open only one index primer at a time. Recap each index tube immediately after use. Extra caps are provided with the kit.
- When adding indexing primers, change tips between **each well**.
- Remove unused indexing primer tubes from the working area.
- Change gloves if gloves come into contact with indexing primers, samples, or probes.

Sealing the Plate

- Always seal the plate with an appropriate plate seal before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate and seal with a rubber roller.
- Apply a new seal every time you cover a plate.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semi-skirted PCR plates. Use Microseal 'B' for shaking, centrifuging, PCR amplification, and long-term storage.
- If you observe droplets hanging from the inside of a sealed plate, centrifuge at 280 × g for 1 minute.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- When instructed to centrifuge the plate, centrifuge at 280 × g for 1 minute.

Handling Reagents

- Tightly recap all reagent tubes immediately after use to limit evaporation and prevent contamination.

- Return reagents to the recommended storage conditions when they are no longer needed for the procedure.
- Stability of the TruSight Oncology 500 Kit has been evaluated and performance demonstrated for up to eight uses of the kit.
- Master mix preparation tables include volume overage to ensure that there is sufficient volume per sample.

Adapter Sets

- The TruSight Oncology 500 Kit includes UMI1 adapters and SUA1 adapters. UMI1 adapters are for use with DNA samples and SUA1 adapters are for use with RNA samples. Do not use SUA1 adapters for DNA libraries. Do not use UMI1 adapters for RNA libraries.

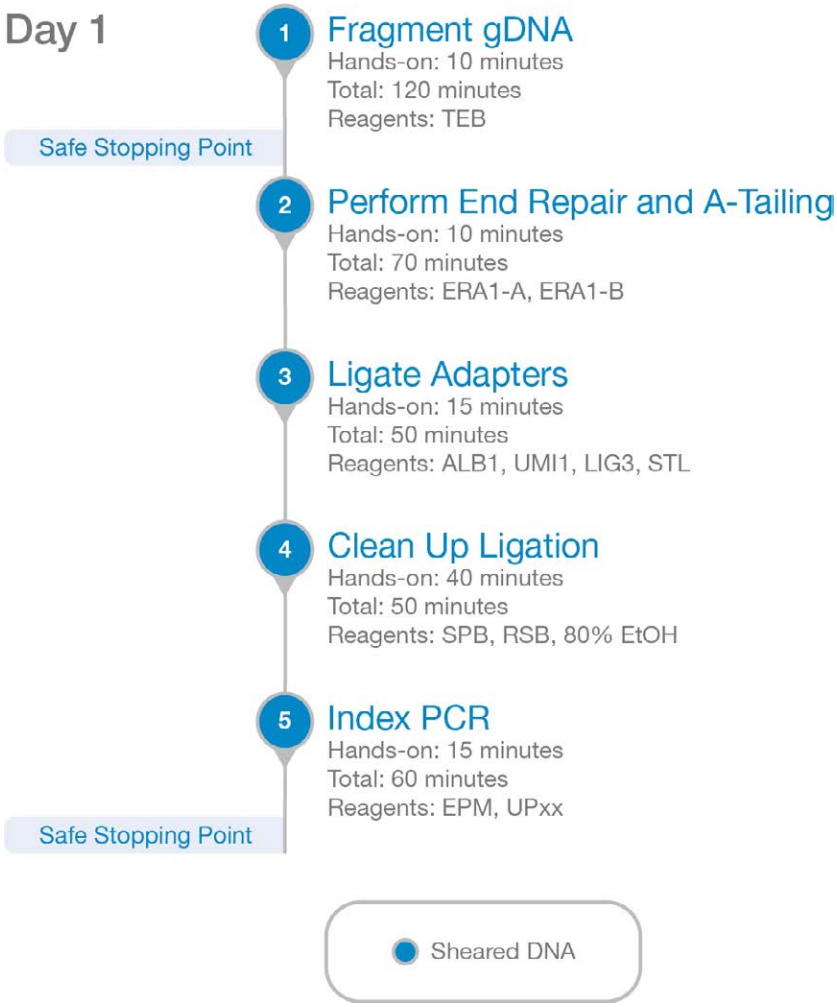
Handling Beads

- When mixing beads with a pipette:
  - Use a suitable pipette and tip size for the volume you are mixing (for example, use a P200 for volumes from 20 µl to 200 µl).
  - Adjust the volume setting to ~50–75% of your sample volume.
  - Pipette with a slow, smooth action.
  - Avoid aggressive pipetting, splashing, and introducing bubbles.
  - Position the pipette tip above the pellet and dispense directly into the pellet to release beads from the well or tube.
  - Make sure that the bead pellet is fully in solution. (For example, for SMB pellets, the solution should look dark brown and have a homogenous consistency.)
- Make sure that beads are at room temperature before use.
- Mix beads for 1 minute before use to ensure homogeneity.
- If beads are aspirated into the pipette tips during magnetic separation steps, dispense back to the same well of the plate on the magnetic stand. Then wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Dispense liquid directly onto the bead pellet so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

Library Prep DNA Only Workflow

The following diagram illustrates the recommended DNA only library preparation workflow using the TruSight Oncology 500 Kit. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on eight DNA samples. Times include degassing the Covaris ultrasonicator.

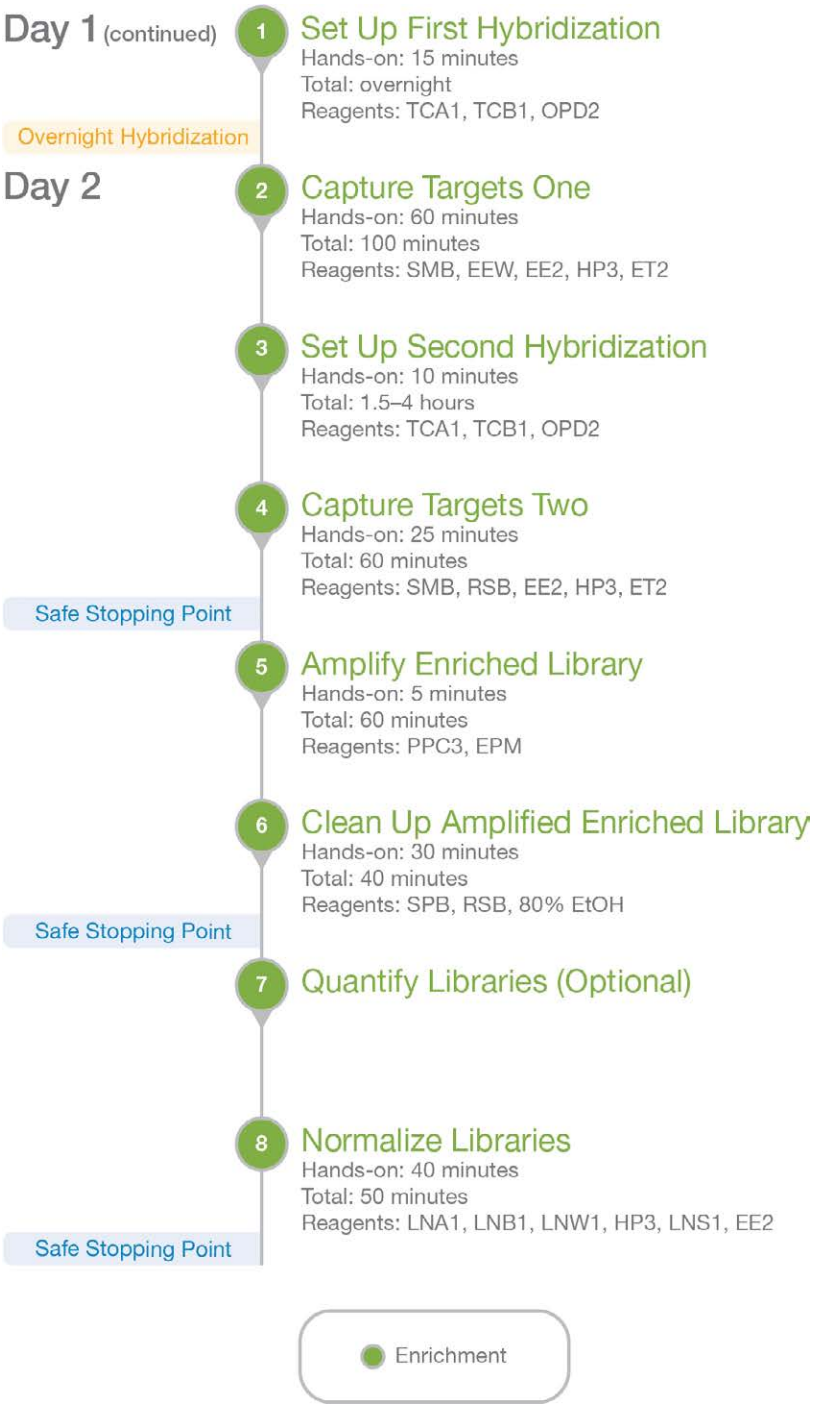
Figure 1 TruSight Oncology 500 Kit DNA Only Workflow (Part 1)



Enrichment DNA Only Workflow

The following diagram illustrates the recommended enrichment DNA only workflow using the TruSight Oncology 500 Kit. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on eight DNA samples.

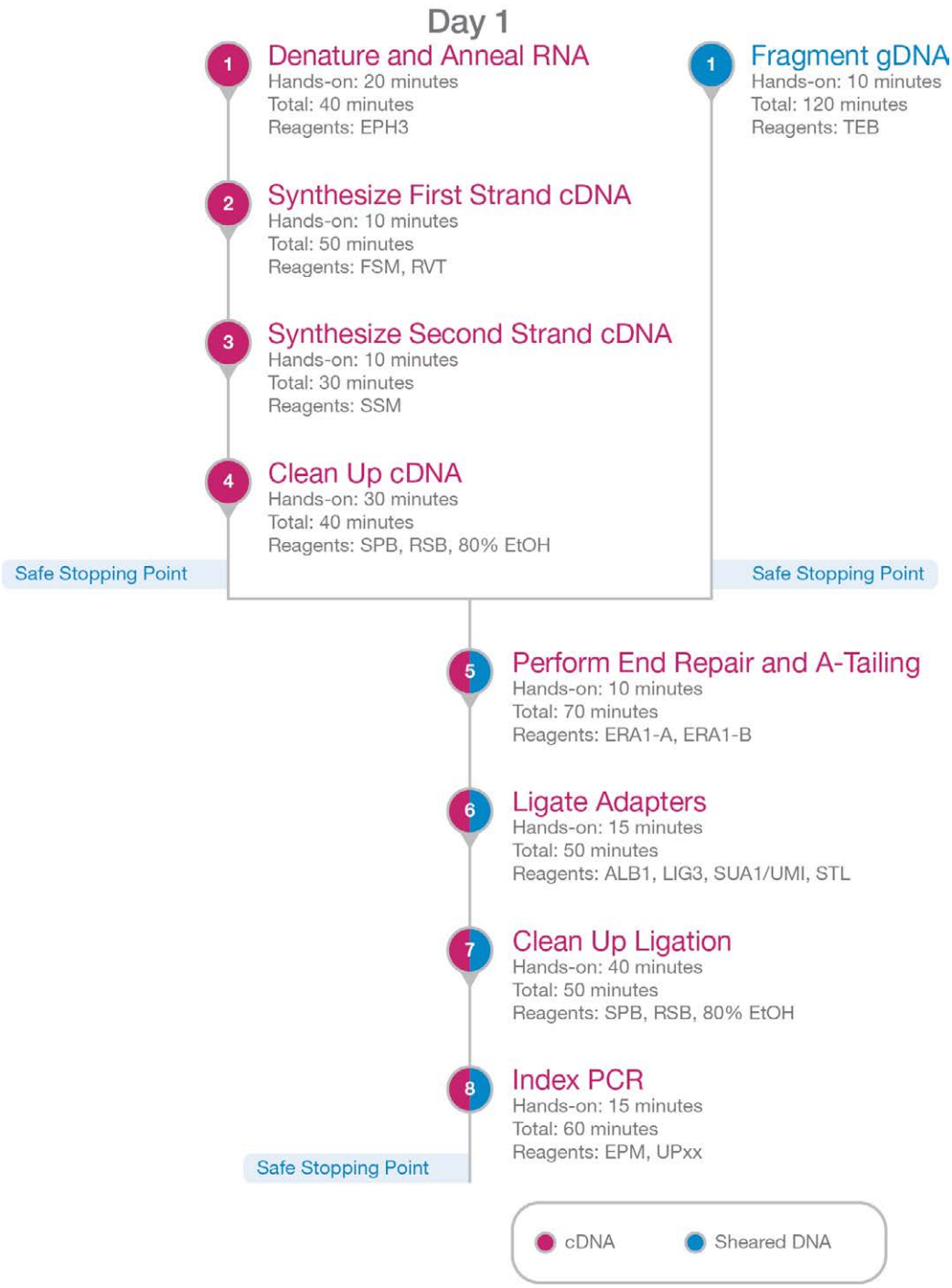
Figure 2 TruSight Oncology 500 Kit DNA Only Workflow (Part 2)



Library Prep DNA and RNA Workflow

The following diagram illustrates the recommended library preparation DNA and RNA workflow using a TruSight Oncology 500 Kit. RNA and DNA libraries can be prepared simultaneously. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on eight DNA samples and eight RNA samples. Times include degassing the Covaris ultrasonicator.

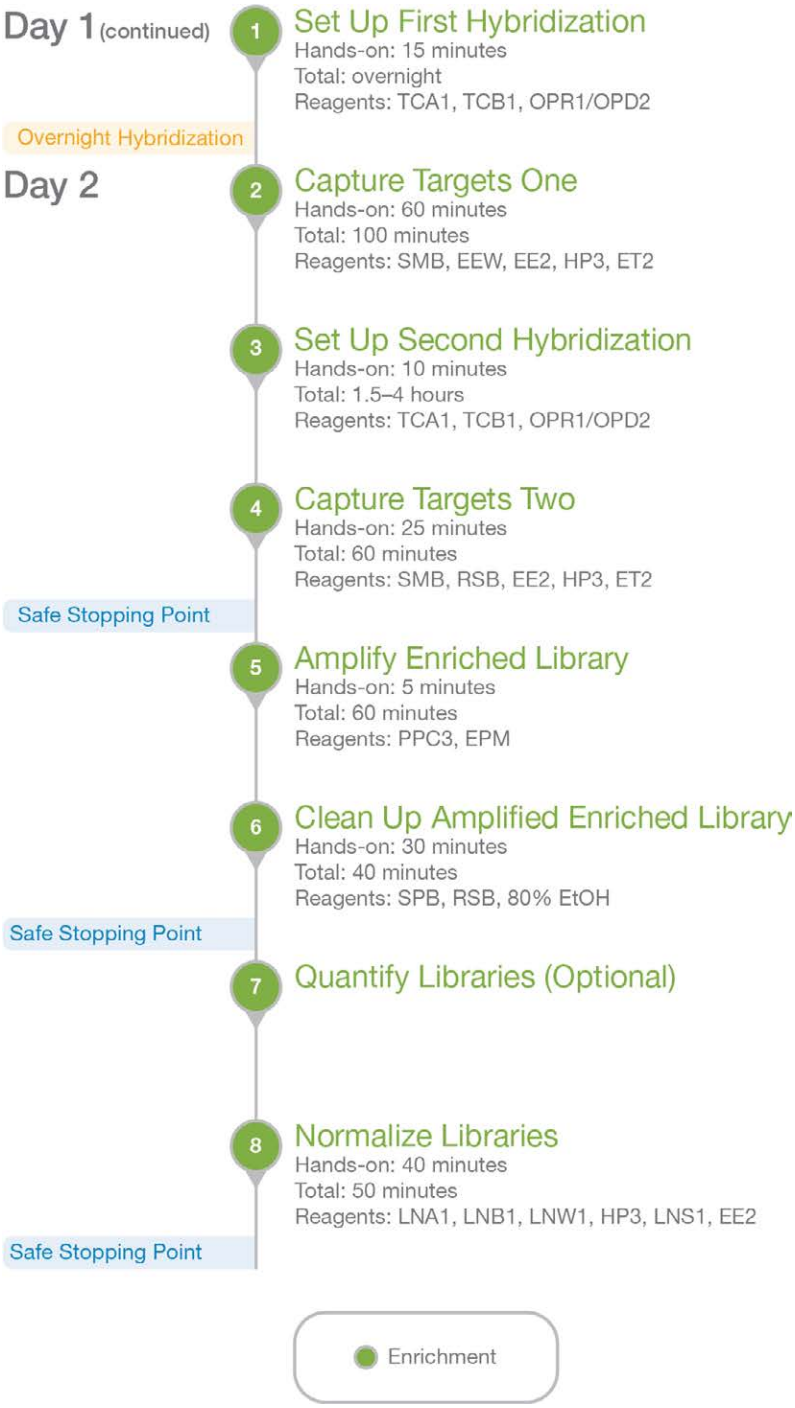
Figure 3 TruSight Oncology 500 Kit DNA and RNA Workflow (Part 1)



Enrichment DNA and RNA Workflow

The following diagram illustrates the recommended enrichment DNA and RNA workflow using a TruSight Oncology 500 Kit. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on eight DNA samples and eight RNA samples.

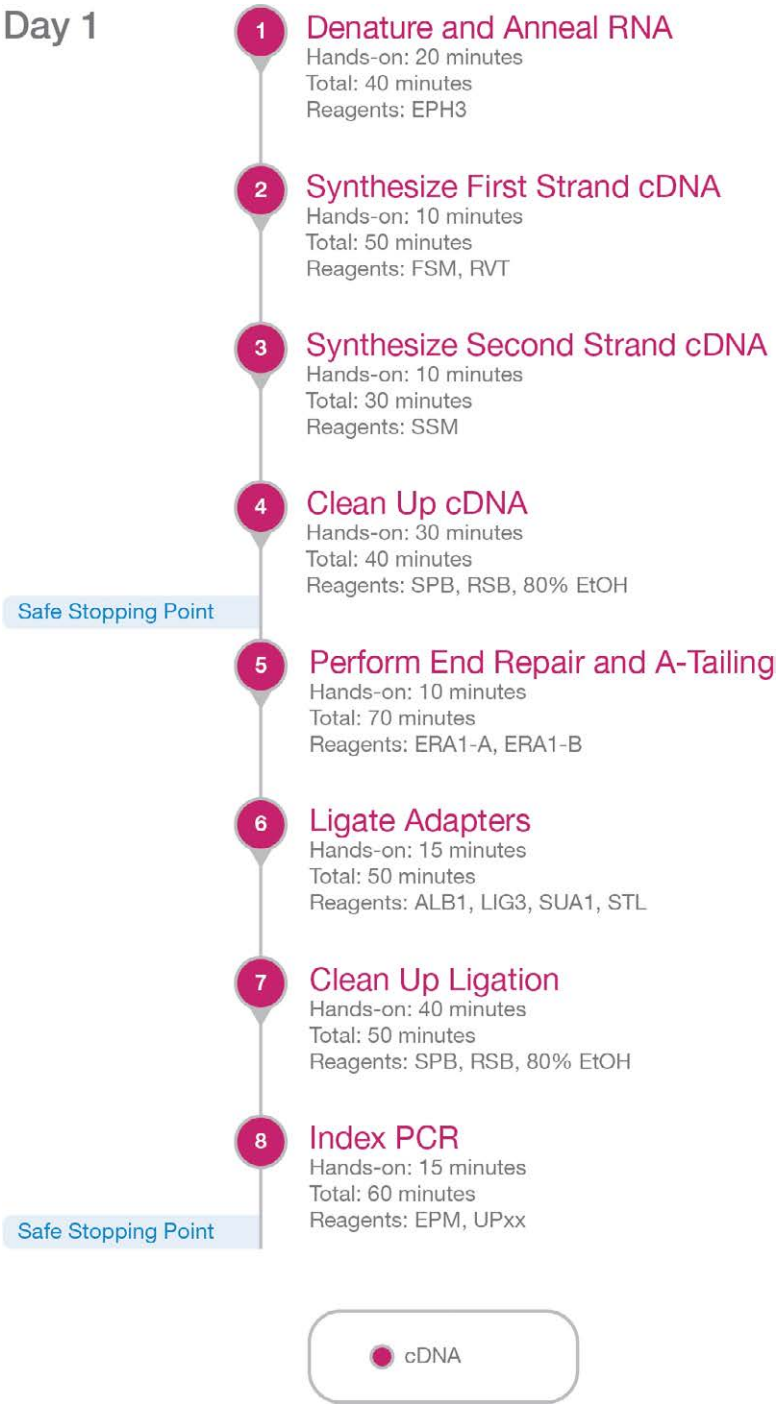
Figure 4 TruSight Oncology 500 Kit DNA and RNA Workflow (Part 2)



Library Prep RNA Only Workflow

The following diagram illustrates the recommended RNA only library preparation workflow using the TruSight Oncology 500 Kit. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on 8 RNA samples.

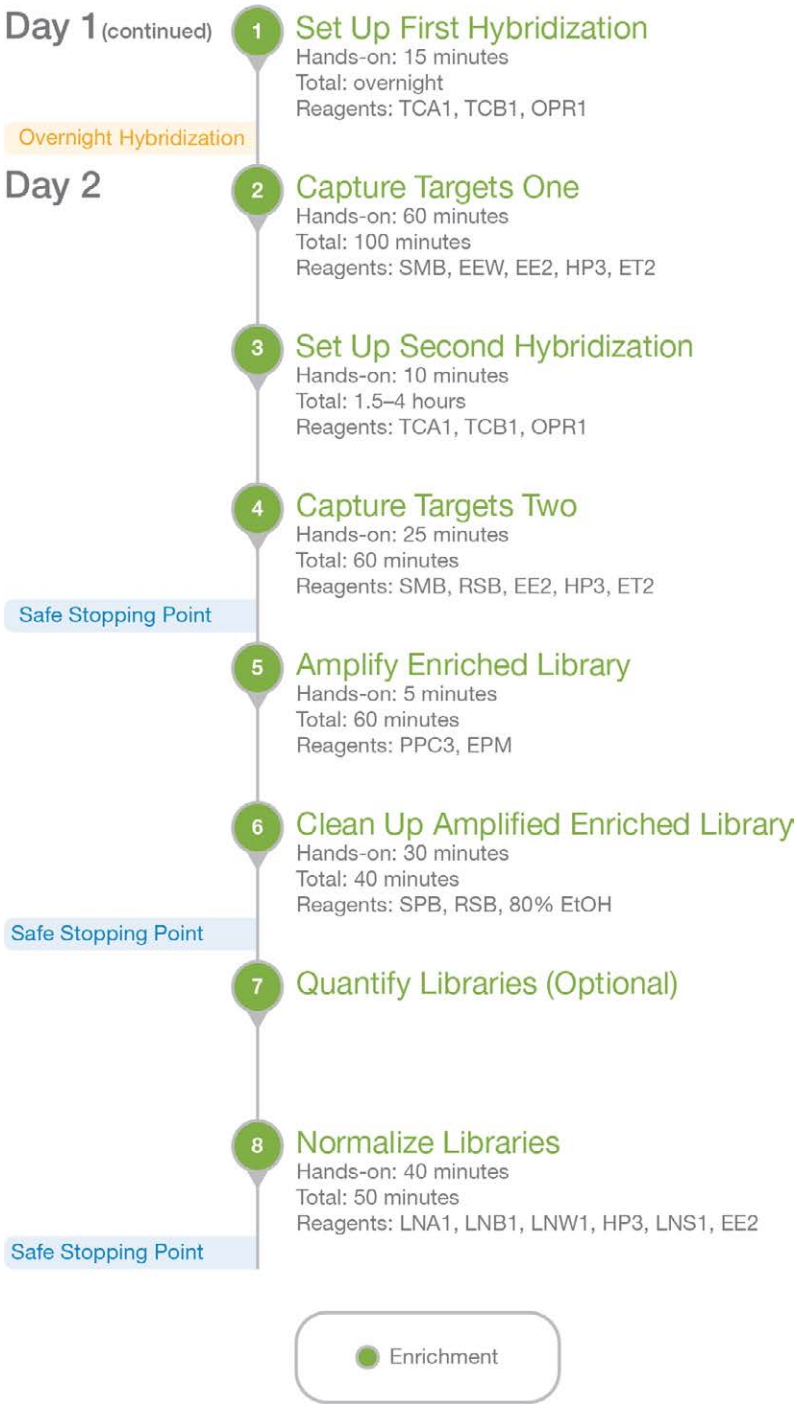
Figure 5 TruSight Oncology 500 Kit RNA Only Workflow (Part 1)



Enrichment RNA Only Workflow

The following diagram illustrates the recommended enrichment RNA only workflow using the TruSight Oncology 500 Kit. Safe stopping points are marked between steps. Hands-on and total times are approximate and based on 8 RNA samples.

Figure 6 TruSight Oncology 500 Kit RNA Only Workflow (Part 2)



Denature and Anneal RNA

During this process, purified RNA is denatured and primed with random hexamers in preparation for cDNA synthesis.

If you are working with only purified DNA, proceed directly to *Fragment gDNA* on page 16.

Consumables

- EPH3 (Elute, Prime, Fragment High Mix 3) (red cap)
- FSM (First Strand Synthesis Mix) (red cap)
- RVT (Reverse Transcriptase) (red cap)
- Nuclease-free water
- 96-well PCR plate
- Microseal 'B' adhesive seals

CAUTION

The following procedures require an RNase- and DNase-free environment. Thoroughly decontaminate your work area with an RNase-inhibiting cleaner. Make sure that you use RNA-dedicated equipment.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EPH3	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
FSM	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
RVT	-25°C to -15°C	Keep on ice. Centrifuge briefly.

2 Thaw RNA samples on ice.

3 Qualify and quantify the samples. See *DNA/RNA Input Recommendations* on page 1.

4 Dilute a minimum of 40 ng RNA sample in RNase/DNase-free water for a final volume of 8.5 µl.

5 Save the following programs on the thermal cycler:
- For FFPE or fragmented RNA, save the LQ-RNA program.
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 17 µl
  - 65°C for 5 minutes
  - Hold at 4°C
- For cell line or intact RNA, save the HQ-RNA program.
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 17 µl
  - 94°C for 8 minutes
  - Hold at 4°C

6 Label a new 96-well PCR plate CF (cDNA Fragments).

Procedure

1 Combine the following volumes in a microcentrifuge tube to prepare the FSM+RVT Master Mix:

Master Mix Component	3 Samples (µl)	8 Samples (µl)	16 Samples (µl)	24 Samples (µl)
FSM	27	72	144	216
RVT	3	8	16	24

- Prepare a minimum of 3 samples.
- Discard any remaining master mix after use.

2 Pipette to mix.

3 Place the FSM+RVT Master Mix on ice until *Synthesize First Strand cDNA* on page 13.

4 Add 8.5 µl of each purified RNA sample to the corresponding well of the CF PCR plate.

5 Add 8.5 µl EPH3 to each well.

6 Apply Microseal 'B' and shake the plate at 1200 rpm for 1 minute.

7 Place on the preprogrammed thermal cycler and run the LQ-RNA or HQ-RNA program.

Synthesize First Strand cDNA

This process reverse transcribes the RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase.

Consumables

- FSM+RVT Master Mix
- Microseal 'B' adhesive seals

Preparation

1 Save the following 1stSS program on the thermal cycler with a heated lid:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 25 µl
- 25°C for 10 minutes
- 42°C for 15 minutes
- 70°C for 15 minutes
- Hold at 4°C

Procedure

1 Remove the CF PCR plate from the thermal cycler.

2 Pipette FSM+RVT Master Mix to mix.

3 Add 8 µl FSM+RVT Master Mix to each well.

4 Pipette 5 times to mix.

5 Apply Microseal 'B' and shake the plate at 1200 rpm for 1 minute.

6 Place on the preprogrammed thermal cycler and run the 1stSS program.

7 If you are also preparing DNA libraries, you can begin to fragment gDNA while the 1stSS program is running. Refer to *Fragment gDNA* on page 16.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes double-stranded cDNA.

Consumables

- SSM (Second Strand Mix) (red cap)
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
SSM	-25°C to -15°C	Thaw to room temperature. Invert 10 times to mix. Centrifuge briefly.

2 Save the following 2ndSS program on the thermal cycler with a heated lid. If the lid temperature cannot be set to 30°C, turn off the preheated lid heat option:
   - Choose the preheat lid option and set to 30°C
   - Set the reaction volume to 50 µl
   - 16°C for 25 minutes
   - Hold at 4°C

Procedure

1 Remove the CF PCR plate from the thermal cycler.

2 Add 25 µl SSM to each well.

3 Apply Microseal 'B' and shake the plate at 1200 rpm for 1 minute.

4 Place on the preprogrammed thermal cycler and run the 2ndSS program.

Clean Up cDNA

This process uses SPB to purify the cDNA from unwanted reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well MIDI plates (1–2)
- [Optional] 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw at room temperature and vortex before use.
SPB	2°C to 8°C	Bring to room temperature for at least 30 minutes.

2 Label a new 96-well MIDI plate BIND1.

3 Select one of the following plate options.
- To continue with library prep immediately after cleaning up cDNA, label a new 96-well MIDI plate PCF (Purified cDNA Fragments).
- To store the plate after this step, use a 96-well PCR plate.

4 Prepare fresh 80% EtOH.

Procedure

Bind

1 Remove the CF PCR plate from the thermal cycler.

2 Vortex SPB for 1 minute to resuspend the beads.

3 Add 90 µl SPB to each well of the BIND1 MIDI plate.

4 Transfer 50 µl of each sample from the CF PCR plate to the corresponding well of the BIND1 MIDI plate.

5 Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

Wash

1 Place the BIND1 MIDI plate on a magnetic stand for 5 minutes.

2 Remove and discard all supernatant from each well.

3 Wash beads as follows.

a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
b Wait 30 seconds.
c Remove and discard all supernatant from each well.

4 Wash beads a second time.

5 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove the BIND1 MIDI plate from the magnetic stand.

2 Add 22 µl RSB to each well.

3 Apply Microseal 'B' and shake at 1500 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 20 µl eluate from each well of the BIND1 MIDI plate to the corresponding well of the PCF plate.

7 Add 30 µl RSB to each well of the PCF plate, and then pipette at least 10 times to mix.

8 Select one of the following options based on sample type:
- **DNA and RNA samples**— Proceed to *Fragment gDNA* on page 16 or follow instructions at the safe stopping point. Purified cDNA fragments and sheared DNA samples can be stored in the same plate. Make sure to label wells. See the preparation section in *Fragment gDNA* for more information.
- **RNA samples only**— If you are processing sample from RNA only, and not stopping at the safe stopping point, proceed to *Perform End Repair and A-Tailing* on page 17.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PCF PCR plate, and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Fragment gDNA

This process fragments gDNA to a 90–250 bp fragment size using the Covaris E220*evolution*, LE220, or ME220 Focused-ultrasonicator. Covaris shearing generates dsDNA fragments with 3' and 5' overhangs.

Consumables

- TEB (TE Buffer)
- Covaris 8 microTUBE Strip with foil seals
- 96-well MIDI plate
- **[Optional]** 96-well PCR plate

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
TEB	2°C to 8°C	Bring to room temperature. Invert to mix.

2 Turn on and set up the Covaris instrument according to manufacturer guidelines. This instrument requires ~1 hour to de-gas.

3 Choose one of the following plate options:
- To continue with library prep immediately after shearing gDNA, use a new 96-well MIDI plate.
- To store sheared gDNA after this step, use a 96-well PCR plate.
- To process gDNA and cDNA samples simultaneously, continue to use the PCF plate from *Clean Up cDNA* on page 14.

4 Label the plate LP (Library Preparation).

5 Thaw gDNA samples at room temperature.

6 Invert to mix.

7 See *DNA Input Recommendations* on page 1 to qualify or quantify samples.

8 Dilute a minimum of 40 ng of each purified DNA sample in TEB for a final volume of 12 µl.

Procedure

1 Add 12 µl of each diluted, purified gDNA sample into a Covaris 8 microTUBE Strip.

2 Add 40 µl TEB to each sample.

3 Pipette to mix.

4 Fill the unused Covaris 8 microTUBE Strip wells with 52 µl water.

5 Seal the microTUBE Strip with the foil seal.

6 Centrifuge briefly.

7 If you are using the Covaris E220*evolution*, LE220, or ME220 model, fragment the gDNA using the following settings.

Setting	E220*evolution*	LE220	ME220
Peak Incident Power	175 watts	450 watts	50 watts
Duty Factor	10%	30%	30%
Cycles per Burst	200	200	1000
Treatment Time	280 seconds	250 seconds	10 seconds
Temperature	7°C	7°C	12°C
Intensifier	yes	N/A	N/A
Other	Intensifier	N/A	Wave guide
Pulse repeats	N/A	N/A	20
Average power	N/A	N/A	15 watts

8 Centrifuge tube strip briefly to collect droplets.

9 Transfer 50 µl of each sheared gDNA sample to the corresponding wells of the LP plate (or PCF plate if you are processing cDNA simultaneously).

- A P20 pipette with fine tips can be used when transferring sheared gDNA sample to the LP plate (pipette 20 µl + 20 µl + 10 µl).

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the LP or PCF plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Perform End Repair and A-Tailing

This process converts the 5' and 3' overhangs resulting from the fragmentation step into blunt ends using an End Repair A-Tailing Master Mix (ERA1).

The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. The 3' ends are A-tailed during this reaction to prevent them from ligating to each other during the adapter ligation reaction.

Consumables

- ERA1-A (End Repair A-tailing Enzyme Mix 1)
- ERA1-B (End Repair A-tailing Buffer 1)
- 1.7 ml microcentrifuge tube
- [Optional] 96-well MIDI plate
- Microseal 'B' adhesive seals

CAUTION

If a PCR plate was used to store the gDNA or cDNA samples, follow the plate transfer instructions in step of the *Preparation* on page 18.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
ERA1-A	-25°C to -15°C	Keep on ice. Centrifuge briefly, and then pipette to mix.
ERA1-B	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly, then pipette to mix. Inspect for precipitates. If present, warm the tube in your hands, then pipette to mix until the crystals are dissolved.

2 Bring sheared gDNA and any cDNA to room temperature.

3 If stored in a 96-well PCR plate, pipette to mix. Then transfer 50 µl of sheared gDNA sample and 50 µl of any cDNA to corresponding wells of a new 96-well MIDI plate.

4 Label the MIDI plate LP2 (Library Preparation 2).

5 Preheat two Hybex incubators with MIDI heat block inserts as follows.
- Preheat the first incubator to 30°C.
- Preheat the second incubator to 72°C.

6 Prepare an ice bucket.

Procedure

1 Combine the following volumes in a microcentrifuge tube to prepare ERA1 Master Mix:

Master Mix Component	3 Samples (µl)	8 Samples (µl)	16 Samples (µl)	24 Samples (µl)
ERA1-B	26	69	138	207
ERA1-A	10	27	54	81

- Prepare a minimum of 3 samples.
- Discard any remaining master mix after use.

2 Pipette 10 times to mix, and then place ERA1 Master Mix on ice.

3 Add 10 µl ERA1 Master Mix to each sample in the LP2 MIDI plate.

4 Apply Microseal 'B' and shake the plate at 1800 rpm for 2 minutes.

5 Incubate at 30°C for 30 minutes.

6 Immediately transfer to another incubator at 72°C and incubate for 20 minutes.

7 Place the plate on ice for 5 minutes.

Ligate Adapters

This process ligates adapters to the ends of the cDNA and/or gDNA fragments. SUA1 adapters are ligated to cDNA fragments only. UMI1 adapters containing unique molecular indexes are ligated to gDNA fragments only.

Consumables

- ALB1 (Adapter Ligation Buffer 1)
- LIG3 (DNA Ligase 3)
- STL (Stop Ligation Buffer)
- SUA1 (Short Universal Adapters 1)
- UMI1 (UMI Adapters v1)
- Microseal 'B' adhesive seals

About Reagents

- ALB1 is highly viscous. Pipette slowly to avoid forming bubbles.
- Make sure to use *UMI1 for DNA libraries only*, and *SUA1 for RNA libraries only*.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
ALB1	-25°C to -15°C	Thaw to room temperature. Vortex ≥ 10 seconds to resuspend. Centrifuge briefly.
LIG3	-25°C to -15°C	Keep on ice. Centrifuge briefly, and then pipette to mix.
STL	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
SUA1	-25°C to -15°C	Thaw to room temperature. Vortex ≥ 10 seconds to resuspend. Centrifuge briefly.
UMI1	-25°C to -15°C	Thaw to room temperature. Vortex ≥ 10 seconds to resuspend. Centrifuge briefly.

Procedure

1 Add 60 µl ALB1 to each well.

2 Add 5 µl LIG3 to each well.

3 Add the appropriate adapters to each well.
   - For DNA libraries only, add 10 µl UMI1.
   - For RNA libraries only, add 10 µl SUA1.

4 Apply Microseal ‘B’ and shake the plate at 1800 rpm for 2 minutes.

5 Incubate at room temperature for 30 minutes.

6 Add 5 µl STL to each well.

7 Apply Microseal ‘B’ and shake the plate at 1800 rpm for 2 minutes.

Clean Up Ligation

This process uses SPB to purify the gDNA and cDNA fragments and remove unwanted products, such as unligated adapters.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the suspension.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
RSB	2°C to 8°C -25°C to -15°C	Bring to room temperature. If at -25°C to -15°C, thaw to room temperature and vortex before use.
SPB	2°C to 8°C	Bring to room temperature for at least 30 minutes. Vortex for 1 minute before use.

2 Label a new 96-well PCR plate LS (Library Samples).

3 Prepare fresh 80% EtOH.

Procedure

Bind

1 Vortex SPB for 1 minute to resuspend the beads.

2 Add 112 µl SPB to each well of the LP2 MIDI plate.

3 Apply Microseal ‘B’ and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 5 minutes.

Wash

1 Place the LP2 MIDI plate on the magnetic stand for 10 minutes.

2 Remove and discard all supernatant from each well.

3 Wash beads as follows.

   a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.
   c Remove and discard all supernatant from each well.

4 Wash beads a **second** time.

5 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove from the magnetic stand.

2 Add 27.5 µl RSB to each well.

3 Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 25 µl of each eluate from the LP2 MIDI plate to the corresponding well of the LS PCR plate.

Index PCR

In this step, library fragments are amplified using primers that add index sequences for sample multiplexing. The resulting product contains the complete library of DNA fragments flanked by index sequences and adapters required for cluster generation.

Consumables

- EPM (Enhanced PCR Mix)
- UPxx (Unique Index Primer)
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Vortex to resuspend. Centrifuge briefly.
UPxx	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.

2 Assign one UPxx index primer per library (xx = index primer number).

When sequencing multiple libraries on a single flow cell, assign a different indexing primer to each sample library. Record sample layout orientation and the indexes for each.

NOTE

See the *NextSeq System Denature and Dilute Libraries Guide (document # 15048776)* for guidelines on the number of libraries and possible DNA/RNA combinations per sequencing run.

3 If you sequence DNA and RNA libraries together, make sure that the two library types contain different index primers. For example, if DNA libraries contain index primer UP01, select a different UPxx for RNA libraries.

4 For low-plex sequencing runs, use at least three libraries containing one of the following combinations to provide sufficient index diversity.
   - [UP01,UP02,UP03]
   - [UP04,UP05,UP06]
   - [UP07,UP08,UP09]
   - [UP10,UP11,UP12]

   For example, the first library is assigned UP01, the second library UP02, and the third library UP03.

5 In the post-amp area, save the following I-PCR program on the thermal cycler with a heated lid:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 30 seconds
   - 15 cycles of:
     - 98°C for 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C

Procedure

1 Add 5 µl indexing primer (UPxx) to each well of the LS PCR plate. Apply a new tube cap.

2 Add 20 µl EPM to each well.

3 Apply Microseal 'B' and shake the plate at 1200rpm for 1 minute.

CAUTION

Perform the following steps in a post-amp area to prevent amplification product carryover.

4 Briefly centrifuge at 280 × g.

5 Place on the preprogrammed thermal cycler and run the I-PCR program.

6 Relabel the plate ALS (Amplified Library Samples).

7 Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the ALS plate and store at -25°C to -15°C for up to 30 days.

Set Up First Hybridization

During this process, a pool of oligos specific to 55 genes hybridize to RNA libraries, and a pool of oligos specific to 523 genes hybridize to DNA libraries prepared in *Index PCR* on page 21. Enrichment of targeted regions requires two hybridization steps. In this step, the first hybridization, oligos hybridize to the DNA and/or RNA libraries overnight (8—24 hours).

Consumables

- OPD2 (Oncology Probes DNA 2) (yellow cap)
- OPR1 (Oncology Probes RNA 1) (red cap)
- TCA1 (Target Capture Additives 1)
- TCB1 (Target Capture Buffer 1)
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Use OPD2 for DNA libraries only (yellow cap).
- Use OPR1 for RNA libraries only (red cap).

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
OPD2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
TCA1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
TCB1	2°C to 8°C	Thaw to room temperature. Centrifuge briefly, then pipette to mix. Inspect for precipitates. If present, warm the tube in your hands, then pipette to mix until the crystals are dissolved.

2 If the ALS plate was stored at -25°C to -15°C, thaw at room temperature, pipette to mix, and then centrifuge.

3 Label a new 96-well PCR plate HYB1 (Hybridization 1).

4 Save the following HYB1 program on the thermal cycler with a heated lid:

- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 95°C for 10 minutes
- 85°C for 2.5 minutes
- 75°C for 2.5 minutes
- 65°C for 2.5 minutes
- Hold at 57°C

Procedure

1 Transfer 20 µl of each library to the HYB1 PCR plate.

2 Add 15 µl TCB1 to each well.

3 Add 10 µl TCA1 to each well.

4 Add the appropriate probe.
- For DNA libraries, add 5 µl OPD2 (yellow cap).
- For RNA libraries, add 5 µl OPR1 (red cap).

5 Apply Microseal 'B' and shake the plate at 1200 rpm for 2 minutes.

6 Place on the preprogrammed thermal cycler and run the HYB1 program. Hybridize for 8—24 hours (overnight) at 57°C.

Capture Targets One

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted library DNA regions of interest. Three heated washes using EEW remove nonspecific DNA binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- EE2 (Enrichment Elution 2)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
EEW	-25°C to -15°C	Thaw to room temperature. Vortex for 1 minute to resuspend.
ET2	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
SMB	2°C to 8°C	Bring to room temperature for 30 minutes. If the bead pellet is present, pipette up and down to release the pellet, and then vortex to resuspend.

2 Preheat a Hybex incubator with MIDI heat block insert to 57°C.

3 Label a new 96-well MIDI plate CAP1 (Capture 1).

4 Label a new 96-well PCR plate ELU1 (Elution 1).

Procedure

Bind

1 Remove the HYB1 PCR plate from the thermal cycler.

2 Vortex SMB for 1 minute to resuspend the beads.

3 Add 150 µl SMB to each well of the CAP1 MIDI plate.

4 Transfer 50 µl of each library from the HYB1 PCR plate to the corresponding well of the CAP1 MIDI plate.

5 Apply Microseal 'B' to the CAP1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

6 Incubate in a Hybex incubator at 57°C for 25 minutes.

7 Place on a magnetic stand for 2 minutes.

8 While on the magnetic stand, use a pipette to remove and discard the supernatant.

Wash

1 Wash beads as follows.

a Remove the CAP1 MIDI plate from the magnetic stand.
b Add 200 µl EEW to each well.
c Pipette to mix 10 times. Use clean tips for each library.
d Apply Microseal 'B' and shake the plate at 1800 rpm for 4 minutes.
If the bead pellet is still present, remove the Microseal and pipette to mix, making sure that all beads are

resuspended. Apply a new Microseal 'B'.

e Incubate in a Hybex incubator at 57°C for 5 minutes.

f Place on a magnetic stand for 2 minutes.

g While on the magnetic stand, use a pipette to remove and discard the supernatant from each well.

2 Wash beads a **second** time.

3 Wash beads a **third** time.

4 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	8 Libraries (µl)	16 Libraries (µl)	24 Libraries (µl)
EE2	95	171	342	513
HP3	5	9	18	27

- Prepare for a minimum of 3 libraries.
- Discard remaining mix after use.

2 Vortex briefly to mix.

3 Remove the CAP1 MIDI plate from the magnetic stand.

4 Carefully add 17 µl EE2+HP3 Elution Mix to each sample pellet.

5 Apply Microseal 'B' and shake the plate at 1800 rpm for 2 minutes.

6 Place on a magnetic stand for 2 minutes.

7 Carefully transfer 15 µl eluate from each well of the CAP1 MIDI plate to the ELU1 PCR plate.

8 Add 5 µl ET2 to each eluate in the ELU1 PCR plate.

9 Apply Microseal 'B' to the ELU1 PCR plate and shake the plate at 1200 rpm for 2 minutes.

Set Up Second Hybridization

This step binds targeted regions of the enriched RNA and/or DNA libraries with capture probes a second time. The second hybridization ensures high specificity of the captured regions. To ensure optimal enrichment of libraries, perform the second hybridization step for a minimum of 1.5 hours to a maximum of 4 hours.

Consumables

- OPD2 (Oncology Probes DNA 2) (yellow cap)
- OPR1 (Oncology Probes RNA 1) (red cap)
- TCA1 (Target Capture Additives 1)
- TCB1 (Target Capture Buffer 1)
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Use OPR1 for RNA libraries only (red cap).
- Use OPD2 for DNA libraries only (yellow cap).

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
OPD2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
TCA1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
TCB1	2°C to 8°C	Thaw to room temperature. Centrifuge briefly, then pipette to mix. Inspect for precipitates. If present, warm the tube in your hands, then pipette to mix until the crystals are dissolved.

2 Save the following HYB2 program on the thermal cycler with a heated lid:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 95°C for 10 minutes
- 85°C for 2.5 minutes
- 75°C for 2.5 minutes
- 65°C for 2.5 minutes
- Hold at 57°C

Procedure

1 Add 15 µl TCB1 to each well of the ELU1 PCR plate.

2 Add 10 µl TCA1 to each well.

3 Add the appropriate probe to each well.
- For DNA libraries, add 5 µl OPD2 (yellow cap).
- For RNA libraries, add 5 µl OPR1 (red cap).

4 Apply Microseal 'B' and shake the plate at 1200 rpm for 2 minutes.

5 Place on the preprogrammed thermal cycler and run the HYB2 program. Hybridize at 57°C for 1.5–4 hours.

Capture Targets Two

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. RSB is used to rinse the captured libraries and remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

- EE2 (Enrichment Elution 2)
- ET2 (Elute Target Buffer 2)
- HP3 (2 N NaOH)
- RSB (Resuspension Buffer)
- SMB (Streptavidin Magnetic Beads)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
ET2	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw to room temperature and vortex before use.
SMB	2°C to 8°C	Bring to room temperature for 30 minutes. If the bead pellet is present, pipette up and down to release the pellet, and then vortex to resuspend.

2 Preheat a Hybex incubator with MIDI heat block insert to 57°C.

3 Label a new 96-well MIDI plate CAP2 (Capture 2).

4 Label a new 96-well PCR plate ELU2 (Elution 2).

Procedure

Bind

1 Remove the ELU1 PCR plate from the thermal cycler.

2 Vortex SMB for 1 minute to resuspend the beads.

3 Add 150 µl SMB to each well of the CAP2 MIDI plate.

4 Transfer 50 µl of each library from the ELU1 PCR plate to the corresponding well of the CAP2 MIDI plate.

5 Apply Microseal 'B' to the CAP2 MIDI plate and shake the CAP2 MIDI plate at 1800 rpm for 2 minutes.

6 Incubate in a Hybex incubator at 57°C for 25 minutes.

7 Place on a magnetic stand for 2 minutes.

8 While on the magnetic stand, use a pipette to carefully remove and discard the supernatant from each well.

Wash

1 Wash as follows.

   a Remove the CAP2 MIDI plate from the magnetic stand.
   b Add 200 µl RSB to each well.
   c Apply Microseal 'B' and shake the plate at 1800 rpm for 4 minutes.
   d If the bead pellet is still present, remove the Microseal and pipette to mix, making sure that all beads are resuspended. Apply a new Microseal 'B'.
   e Place on a magnetic stand for 2 minutes.
   f While on the magnetic stand, use a pipette to carefully remove and discard the supernatant.

2 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	8 Libraries (µl)	16 Libraries (µl)	24 Libraries (µl)
EE2	95	209	418	627
HP3	5	11	22	33

   - Prepare for a minimum of 3 libraries.
   - Discard remaining mix after use.

2 Vortex to mix.

3 Remove the CAP2 MIDI plate from the magnetic stand.

4 Carefully add 22 µl EE2+HP3 Elution Mix to each sample pellet.

5 Apply Microseal 'B' and shake the CAP2 MIDI plate at 1800 rpm for 2 minutes.

6 Place on a magnetic stand for 2 minutes.

7 Transfer 20 µl eluate from each well of the CAP2 MIDI plate to the ELU2 PCR plate.

8 Add 5 µl ET2 to each eluate in the ELU2 PCR plate.

9 Apply Microseal 'B' to the ELU2 PCR plate and shake the ELU2 PCR plate at 1200 rpm for 2 minutes.

10 Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, store ELU2 plate at -25°C to -15°C for up to 7 days.

Amplify Enriched Library

This step uses primers to amplify enriched libraries.

Consumables

- EPM (Enhanced PCR Mix)
- PPC3 (PCR Primer Cocktail 3)
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Vortex to resuspend. Centrifuge briefly.
PPC3	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.

2 If the ELU2 plate was stored at -25°C to -15°C, thaw at room temperature, pipette to mix, and then centrifuge.

3 Save the following EL-PCR program on the thermal cycler with a heated lid:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 98°C for 30 seconds
- 18 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Procedure

1 Add 5 µl PPC3 to each well of the ELU2 PCR plate.

2 Add 20 µl EPM to each well.

3 Apply Microseal 'B' and shake the plate at 1200 rpm for 2 minutes.

4 Briefly centrifuge at 280 × g.

5 Place on the preprogrammed thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

This step uses SPB (Sample Purification Beads) to purify the enriched library from unwanted reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
SPB	2°C to 8°C	Bring to room temperature for 30 minutes.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw to room temperature and vortex before use.

2 Label a new 96-well MIDI plate BIND2.

3 Label a new 96-well PCR plate PL (Purified Libraries).

4 Prepare fresh 80% EtOH.

Procedure

Bind

1 Remove the ELU2 PCR plate from the thermal cycler.

2 Vortex SPB for 1 minute to resuspend the beads.

3 Add 110 µl SPB to each well of the BIND2 MIDI plate.

4 Transfer 50 µl of each library from the ELU2 PCR plate to the corresponding well of the BIND2 MIDI plate.

5 Apply Microseal 'B' to the BIND2 MIDI plate and shake at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

Wash

1 Place the BIND2 MIDI plate on magnetic stand for 5 minutes.

2 Remove and discard all supernatant from each well.

3 Wash beads as follows.

   a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.
   c Remove and discard all supernatant from each well.

4 Wash beads a **second** time.

5 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove the BIND2 MIDI plate from the magnetic stand.

2 Add 32 µl RSB to each well.

3 Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 30 µl of each eluate from the BIND2 MIDI plate to the corresponding well of the PL PCR plate.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PL plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Quantify Libraries (Optional)

Accurately quantify to make sure that there is sufficient library available for clustering on the flow cell. Use a fluorometric quantification method (user-supplied) to assess the quantity of enriched libraries before library normalization. Efficient bead-based library normalization requires ≥ 3 ng/µl of each library. The AccuClear Ultra High Sensitivity dsDNA Quantitation Kit has been demonstrated to be effective for quantifying libraries in this protocol.

Recommended Guidelines (AccuClear)

1 Combine 6 µl DNA standard with 44 µl RSB to dilute DNA standard to 3 ng/µl.

2 Use RSB as blank.

3 Run the diluted AccuClear DNA standard and blanks in triplicate.

4 Run libraries in single replicates.

5 Determine the average relative fluorescence unit (RFU) for DNA standard and blank.

6 Calculate the following values.
- Average Standard RFU - Average Blank RFU = Normalized Standard RFU
- Library RFU - Average Blank RFU = Normalized RFU for each library

Assess Quantity

Assess the resulting Normalized RFU for each library against the following criteria.

Fluorescence Measurement	Recommendation
≤ Average Blank RFU	Repeat library preparation and enrichment if purified DNA sample meets quantity and quality specifications.
> Average Blank RFU (and) < Normalized Standard RFU	Proceed to *Normalize Libraries*. Note: Using libraries with RFU below the Normalized Standard RFU might not yield adequate sequencing results to confidently call variants that can be present in the sample.
≥ Normalized Standard RFU	Proceed to *Normalize Libraries*.

Normalize Libraries

This process uses bead-based normalization to normalize the quantity of each library to ensure a uniform library representation in the pooled libraries.

Consumables

- EE2 (Enrichment Elution 2)
- HP3 (2 N NaOH)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNS1 (Library Normalization Storage 1)
- LNW1 (Library Normalization Wash 1)
- 1.7 ml microcentrifuge tubes (2)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Aspirate and dispense LNB1 slowly due to the viscosity of the suspension.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
LNA1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
LNB1	2°C to 8°C	Bring to room temperature for at least 30 minutes. Pipette LNB1 pellet up and down to resuspend.
LNS1	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
LNW1	2°C to 8°C	Bring to room temperature. Vortex to resuspend.

2 If the PL plate was stored at -25°C to -15°C, thaw at room temperature, pipette to mix, and then centrifuge.

3 Label a new 96-well MIDI plate BBN (Bead-Based Normalization).

4 Label a new 96-well PCR plate NL (Normalized Libraries).

Procedure

1 Vortex LNB1 for 1 minute, and then pipette 10 times to mix and ensure bead pellet resuspension.

NOTE

It is critical to completely resuspend the bead pellet at the bottom of the tube. Resuspension is essential to achieve consistent cluster density.

2 Combine the following reagents in a new microcentrifuge tube to create LNA1+LNB1 Master Mix:

Master Mix Component	3 Libraries (µl)	8 Libraries (µl)	16 Libraries (µl)	24 Libraries (µl)
LNA1	132	352	704	1056
LNB1	24	64	128	192

3 Vortex to mix.

4 Combine the following reagents in a new microcentrifuge tube to create a fresh EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	8 Libraries (µl)	16 Libraries (µl)	24 Libraries (µl)
EE2	114	304	608	912
HP3	6	16	32	48

5 Vortex to mix.

Bind

1 Vortex LNA1+LNB1 Master Mix.

2 Add 45 µl LNA1+LNB1 Master Mix to each well of the BBN MIDI plate.

3 Add 20 µl of each library from the PL PCR plate to the corresponding well of the BBN MIDI plate.

4 Apply Microseal 'B' and shake at 1800 rpm for 30 minutes.

5 Place the BBN MIDI plate on a magnetic stand for 2 minutes.

6 Remove and discard all supernatant from each well.

Wash

1 Wash as follows.

   a Remove the BBN MIDI plate from the magnetic stand.
   b Add 45 µl LNW1 to each well.
   c Apply Microseal 'B' and shake at 1800 rpm for 5 minutes.
   d Place on a magnetic stand for 2 minutes.
   e Remove and discard all supernatant from each well.

2 Wash beads a **second** time.

3 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Remove the BBN MIDI plate from the magnetic stand.

2 Carefully add 32 µl EE2+HP3 Elution Mix to each well

3 Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.

4 Place BBN MIDI plate on a magnetic stand for 2 minutes.

5 Transfer 30 µl of each eluate from the BBN MIDI plate to the corresponding well of the NL PCR plate.

6 Add 30 µl LNS1 to each library in the NL PCR plate.

7 Pipette up and down to mix.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the NL plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Pool Libraries and Dilute to the Loading Concentration

1 See the denature and dilute libraries guide for your sequencing system to pool, denature, and dilute libraries to the loading concentration.

Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

Kit Contents

Make sure that you have the reagents identified in this section before proceeding to the protocol.

Library prep kit	Catalog #
TruSight Oncology 500 DNA Kit (48 Sample Library Prep Kit Only)	20028213
TruSight Oncology 500 DNA/RNA Bundle (24 Sample Library Prep Kit Only)	20028215

Library prep kit plus NextSeq System reagents	Catalog #
TruSight Oncology 500 DNA NextSeq Kit (48 Sample Library Prep Kit with NextSeq Kit)	20028214
TruSight Oncology 500 DNA/RNA Bundle NextSeq Kit (24 Sample Library Prep Kit with NextSeq Kit)	20028216

Library prep kit plus access to the PierianDx Clinical Genomics Workspace	Catalog #
TruSight Oncology 500 DNA Kit, plus PierianDx (48 Sample Library Prep Kit with PierianDx)	20032624
TruSight Oncology 500 DNA/RNA Bundle Kit, plus PierianDx (24 Sample Library Prep Kit with PierianDx)	20032626

Library prep kit plus NextSeq System reagents, plus access to the PierianDx Clinical Genomics Workspace	Catalog #
TruSight Oncology 500 DNA NextSeq Kit, plus PierianDx (48 Sample Library Prep Kit with NextSeq Kit and PierianDx)	20032625
TruSight Oncology 500 DNA/RNA NextSeq Kit, plus PierianDx (24 Sample Library Prep Kit with NextSeq Kit and PierianDx)	20032627

Library Prep

Box 1 - Library Prep – RNA (Pre-Amp), Store at -25°C to -15°C

DNA/RNA bundle customers receive one box. DNA kit customers do not receive this box.

Quantity	Reagent	Description
1	EPH3	Elute, Prime, Fragment High Mix 3
1	FSM	First Strand Synthesis Mix
1	RVT	Reverse Transcriptase
1	SSM	Second Strand Mix

Box 2- Library Prep (Pre-Amp), Store at -25°C to -15°C

Quantity	Reagent	Description
2	UMI1	UMI Adapters v1

Box 3 - Library Prep (Pre-Amp), Store at -25°C to -15°C

Quantity	Reagent	Description
2	ALB1	Adapter Ligation Buffer 1
2	EPM	Enhanced PCR Mix
2	ERA1-A	End Repair A-tailing Enzyme Mix 1
2	ERA1-B	End Repair A-tailing Buffer 1
2	LIG3	DNA Ligase 3
2	STL	Stop Ligation Buffer
2	SUA1	Short Universal Adapters 1

Box 4 - Library Prep (Pre-Amp), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SPB	Sample Purification Beads	2°C to 8°C
1	TEB	TE Buffer	2°C to 8°C

Box 5 - Library Prep - Unique PCR Index Primers (Pre-Amp), Store at -25°C to -15°C

Quantity	Reagent	Description
1	UP01	Unique Index Primer 01
1	UP02	Unique Index Primer 02
1	UP03	Unique Index Primer 03
1	UP04	Unique Index Primer 04
1	UP05	Unique Index Primer 05
1	UP06	Unique Index Primer 06
1	UP07	Unique Index Primer 07
1	UP08	Unique Index Primer 08
1	UP09	Unique Index Primer 09
1	UP10	Unique Index Primer 10
1	UP11	Unique Index Primer 11
1	UP12	Unique Index Primer 12
1	UP13	Unique Index Primer 13
1	UP14	Unique Index Primer 14
1	UP15	Unique Index Primer 15
1	UP16	Unique Index Primer 16

Enrichment

Box 6 - Enrichment (Post-Amp), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
2	ET2	Elute Target Buffer 2	2°C to 8°C
2	HP3	2 N NaOH	2°C to 8°C
1	LNB1	Library Normalization Beads 1	2°C to 8°C
2	LNS1	Library Normalization Storage 1	2°C to 8°C
2	LNW1	Library Normalization Wash 1	2°C to 8°C
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
2	SPB	Sample Purification Beads	2°C to 8°C
2	TCB1	Target Capture Buffer 1	2°C to 8°C

Box 7 - Enrichment (Post-Amp), Store at -25°C to -15°C

Quantity	Reagent	Description
3	EE2	Enrichment Elution 2
1	EEW	Enhanced Enrichment Wash
2	EPM	Enhanced PCR Mix
1	LNA1	Library Normalization Additives 1
2	PPC3	PCR Primer Cocktail 3
2	TCA1	Target Capture Additives 1

Box 8 - Enrichment (Post-Amp), Store at -25°C to -15°C

DNA/RNA bundle customers receive one box. DNA kit customers receive two of this box.

Quantity	Reagent	Description
1	OPD2	Oncology DNA Probes Master Pool 2

Box 9 - TruSight Oncology 500 Kit Content Set (RNA Only), Store at -25°C to -15°C

DNA/RNA bundle customers receive one box. DNA kit customers do not receive this box.

Quantity	Reagent	Description
1	OPR1	Oncology RNA Probes Master Pool

Consumables and Equipment

Make sure that you have the required consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
[Optional] AccuClear Ultra High Sensitivity dsDNA Quantitation Kit with 1 DNA Standard	Biotium, catalog # 31029
[Optional] AllPrep DNA/RNA FFPE Kit	QIAGEN, catalog # 80234
[Optional] QuantiFluor RNA System	Promega, catalog # E3310
[Optional] Agilent DNA 1000 Kit	Agilent, catalog # 5067–1504
[Optional] Agilent RNA 6000 Nano Kit	Agilent, catalog # 5067–1511
[Optional] Standard Sensitivity RNA Analysis Kit	Agilent, catalog # DNF-471-0500
[Optional] FFPE QC Kit	Illumina, catalog # WG-321-1001
[Optional] DNA Reference Standard	Horizon Diagnostics, catalog # HD753
[Optional] Universal Human Reference RNA	Agilent, catalog # 740000
8 microTUBE Strip (12) for LE220 and E220 *evolution*	Covaris, part # 520053

Consumable	Supplier
8 microTUBE-50 AFA Fiber H Slit Strip V2 (for use with ME220)	Covaris, part # 520240
Rack E220 *evolution* 8 microTUBE Strip adapter (for use with E220 *evolution*)	Covaris, part # 500430
Rack 12 place 8 microTUBE Strip adapter (for use with LE220)	Covaris, part # 500191
Rack 8 microTUBE Strip V2 (for use with ME220)	Covaris, part # 500518
1.7 ml microcentrifuge tubes, nuclease-free	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
20 µl aerosol resistant pipette tips	General lab supplier
200 µl aerosol resistant pipette tips	General lab supplier
1 ml aerosol resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859
96-well PCR plates, 0.2 ml (polypropylene)	General lab supplier
[Optional] 96-well microplate, black, flat, clear bottom	Corning, part # 3904
Nuclease-free reagent reservoirs (PVC, disposable trough)	VWR, part # 89094-658
Microseal 'B' adhesive seal (adhesive plate seal)	Bio-Rad, part # MSB-1001
RNase/DNase-free water	General lab supplier
Nuclease-free water	General lab supplier
Ethanol (200 proof for molecular biology)	Sigma-Aldrich, part # E7023

Equipment (Pre-Amp)

Equipment	Supplier
Thermal Cycler	General lab supplier
Heat block (1.5 ml microcentrifuge tube)	General lab supplier
(2) Heat blocks (Hybex incubator, heating base)	SciGene, catalog # • 1057-30-O (115 V) or • 1057-30-2 (230 V)
(2) MIDI heat block inserts (for use with Hybex)	Illumina, catalog # BD-60-601
Tabletop centrifuge (plate centrifuge)	General lab supplier
Microcentrifuge (1.5 ml tubes)	General lab supplier
Magnetic stand-96	Thermo Fisher, catalog # AM10027
Vortexer	General lab supplier
Plate shaker (BioShake XP)	Q Instruments, part # 1808-0505
Covaris Focused-ultrasonicator	• Covaris, part # 500219 (model LE220) or • Covaris, part # 500429 (model E220 *evolution*) or • Covaris, part # 500506 (model ME220)

Equipment	Supplier
[Optional] 8 microTUBE Strip Prep Station for LE220 and E220 *evolution*	Covaris, part # 500327
[Optional] Rack Loading Station (for use with ME220 micro TUBE-50 AFA Fiber H Slit Strip V2)	Covaris, part # 500523
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Fragment Analyzer Automated CE System	Agilent, part # M5310AA or M5311AA

Equipment (Post-Amp)

Equipment	Supplier
Heat block (1.5 ml microcentrifuge tube)	General lab supplier
Heat block (Hybex incubator, 96-well plate)	SciGene, catalog # • 1057-30-O (115 V) or • 1057-30-2 (230 V)
MIDI heat block insert (for use with Hybex)	Illumina, catalog # BD-60-601
Tabletop centrifuge (plate centrifuge)	General lab supplier
Microcentrifuge (1.5 ml tubes)	General lab supplier
Magnetic stand-96	Thermo Fisher, catalog # AM10027
Vortexer	General lab supplier
Plate shaker (BioShake XP)	Q Instruments, part # 1808-0505
Thermal cycler	General lab supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Fragment Analyzer Automated CE System	Agilent, part # M5310AA or M5311AA

Acronyms

Acronym	Definition
1stSS	1st Strand Synthesis
2ndSS	2nd Strand Synthesis
ALS	Amplified Library Samples
BBN	Bead Based Normalization
CAP1	Capture 1
CAP2	Capture 2
cDNA	Complementary DNA
CF	cDNA Fragments
ELU1	Elution 1
ELU2	Elution 2
gDNA	Genomic DNA
HQ-RNA	High-quality RNA
HYB1	Hybridization 1

Acronym	Definition
HYB2	Hybridization 2
LP	Library Preparation
LP2	Library Preparation 2
LQ-RNA	Low-quality RNA
LS	Library Samples
NL	Normalized Libraries
PCF	Purified cDNA Fragments
PL	Purified Libraries

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

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North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
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Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download from support.illumina.com.

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