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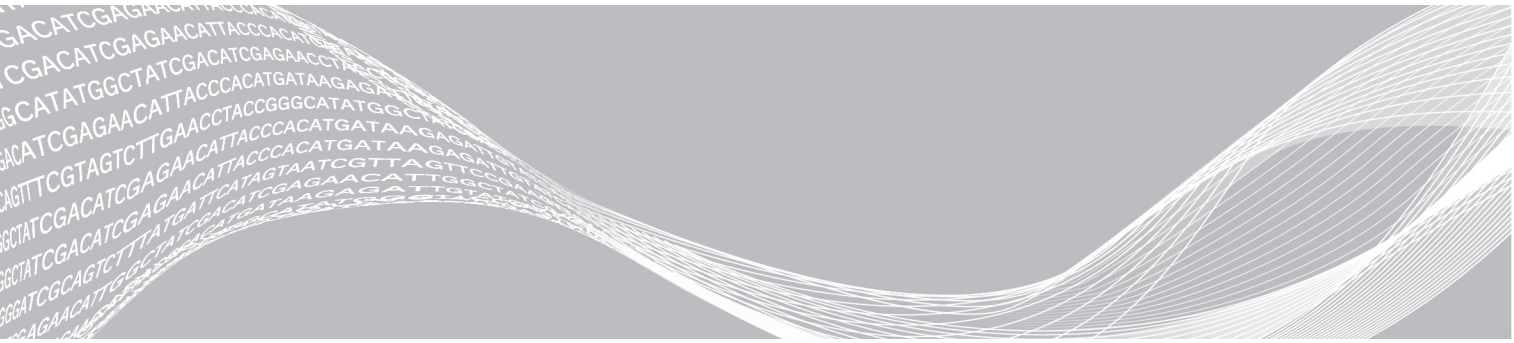
TruSight Tumor 170

Reference Guide



Document # 1000000024091 v02

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January 2019

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Revision History

Document	Date	Description of Change
Document # 1000000024091 v02	December 2018	Moved the denature and dilution steps for pooled libraries to the NextSeq Denature and Dilute Libraries Guide (document # 15048776) and HiSeq Systems Denature and Dilute Libraries Guide (document # 15050107). Added bead handling techniques. Added environmental, health, and safety warnings to relevant sections. Added a section for diluting libraries to the loading concentration. Updated Kit Contents: • Updated the ET2 description. Corrected the following information: • Plate storage instructions in all safe stopping points. • Pipette to mix instructions in the perform first capture section.
Document # 1000000024091 v01	April 2017	Corrected the kits that are used for RNA sample assessment in the RNA/DNA input recommendation section. Clarified that manual normalization is not currently supported by TruSight Tumor 170. Added the Advanced Analytical Technologies Standard Sensitivity RNA Analysis Kit and the Agilent RNA 6000 Nano Kit to the consumables list.
Document # 1000000024091 v00	March 2017	Initial release.

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Chapter 1 Overview

Introduction

The TruSight® Tumor 170 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 Tumor 170 kit allows for preparation of 48 libraries (24 from DNA and 24 from RNA). The kit is optimized to provide high sensitivity and specificity for low-frequency somatic variants across 170 genes. These biomarkers include single nucleotide variants (SNVs), insertions, deletions, multinucleotide variants (MNVs), amplifications, fusions, and splice variants.

Sequencing data can be analyzed with TruSight Tumor 170 software. Software variant calling algorithms provide reports of mutations in full-coding regions of the targeted genes. See the TruSight Tumor 170 support pages for more information.

Product Description

The TruSight Tumor 170 RUO kit consists of library preparation reagents to convert sample nucleic acids to sequenceable libraries. The TruSight Tumor 170 kit also contains the associated TruSight Tumor 170 software. The assay begins with DNA and/or RNA extracted from FFPE tissue as the input sample type. The variant calling algorithms are provided in the TruSight Tumor 170 App. The TruSight Tumor 170 App reports mutations (single nucleotide variants, multinucleotide variants, indels, copy number variants, splice variants, and gene fusions) in full-coding regions of the targeted genes.

RNA/DNA Input Recommendations

The TruSight Tumor 170 assay is optimized for a defined RNA/DNA input range. The optimal range for DNA is from 40 ng to 120 ng total or 3.3 ng/µl to 10 ng/µl. The optimal range for RNA is from 40 ng to 85 ng total or 4.7 ng/µl to 10 ng/µl. Quantify the input RNA/DNA before beginning the protocol. To obtain sufficient nucleic acid material, Illumina recommends isolating 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 compared to other extraction methods tested for this assay.
- Use a fluorometric quantification method that uses RNA/DNA binding dyes such as QuantiFluor® (RNA) or AccuClear™ (DNA).

For optimal performance, assess DNA and RNA sample quality before using the TruSight Tumor 170 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 Reference RNA. The Agilent Universal Reference RNA is an intact RNA sample that should be processed following the intact RNA procedure in *Denature and Anneal RNA* on page 8.
- Qualified FFPE materials from cell line derived xenografts can be used as reference samples.
- Use RNase/DNase-free water as a no template control. Do not sequence the 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 Tumor 170 assay has been optimized to prepare libraries from gDNA that has been fragmented to 90–250 bp (with a peak at ~125 bp). The assay has been optimized using either the Covaris E220 *evolution*™ or LE220 Focused-ultrasonicator with the parameters provided in *Fragment gDNA* on page 12. Fragment size distribution may vary due to differences in sample quality and the sonication instrumentation used with this test. If you are not using the fragmentation method optimized for TruSight Tumor 170 (Covaris E220 *evolution* or LE220), consult the TruSight Tumor 170 support page.

- 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 are using the LE220 Covaris instrument, fill unused Covaris 8 microTUBE Strip wells with 52 µl of water for optimal performance.
- [Optional] Fragment size distribution of sheared samples can be assessed using the Agilent DNA 1000 Kit with the Agilent Bioanalyzer 2100.

Additional Resources

Visit the TruSight Tumor 170 kit support pages on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products, including analysis software. Always check support pages for the latest versions.

The following documentation is available for download from the Illumina website.

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 Tumor 170 Checklist (document # 1000000024092 v02)	Provides a checklist of steps for the experienced user.
TruSight Tumor 170 Consumables & Equipment List (document # 1000000031408 v02)	Provides an interactive checklist of user-provided consumables and equipment.

Chapter 2 Protocol

Introduction

This section describes the TruSight Tumor 170 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 33.
- Some reagents in this protocol have color-coded caps to identify the associated sample type.
  - Blue caps identify reagents used only with genomic DNA (gDNA) samples.
  - Red caps identify reagents used only with RNA or complementary DNA (cDNA) samples.
- Follow the protocol in the order described using the specified parameters.
- Before beginning library preparation, record sample concentration (DNA or RNA) 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 Tumor 170 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. For more information, please see the *Library Prep Workflow* on page 6.
- Day 2: Enrichment, Enriched Library QC Check (Quantify Libraries), Bead-Based Normalization of Enriched Libraries, and Loading of Libraries onto Sequencing Platform. For more information, please see the *Enrichment Workflow* on page 7.

If it is not possible to perform the TruSight Tumor 170 assay workflow according to the preceding schedule, several safe stopping points are specified throughout the protocol to accommodate alternative schedules.

Tips and Techniques

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.
- When adding or transferring samples, change tips between **each well**.
- When adding indexing primers, change tips between **each well**.
- Change gloves if gloves come into contact with indexing primers, samples, or probes.
- Clean work surfaces thoroughly before and after the procedure.
- Remove unused indexing primer tubes from the working area.

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.
- 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.

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.

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).

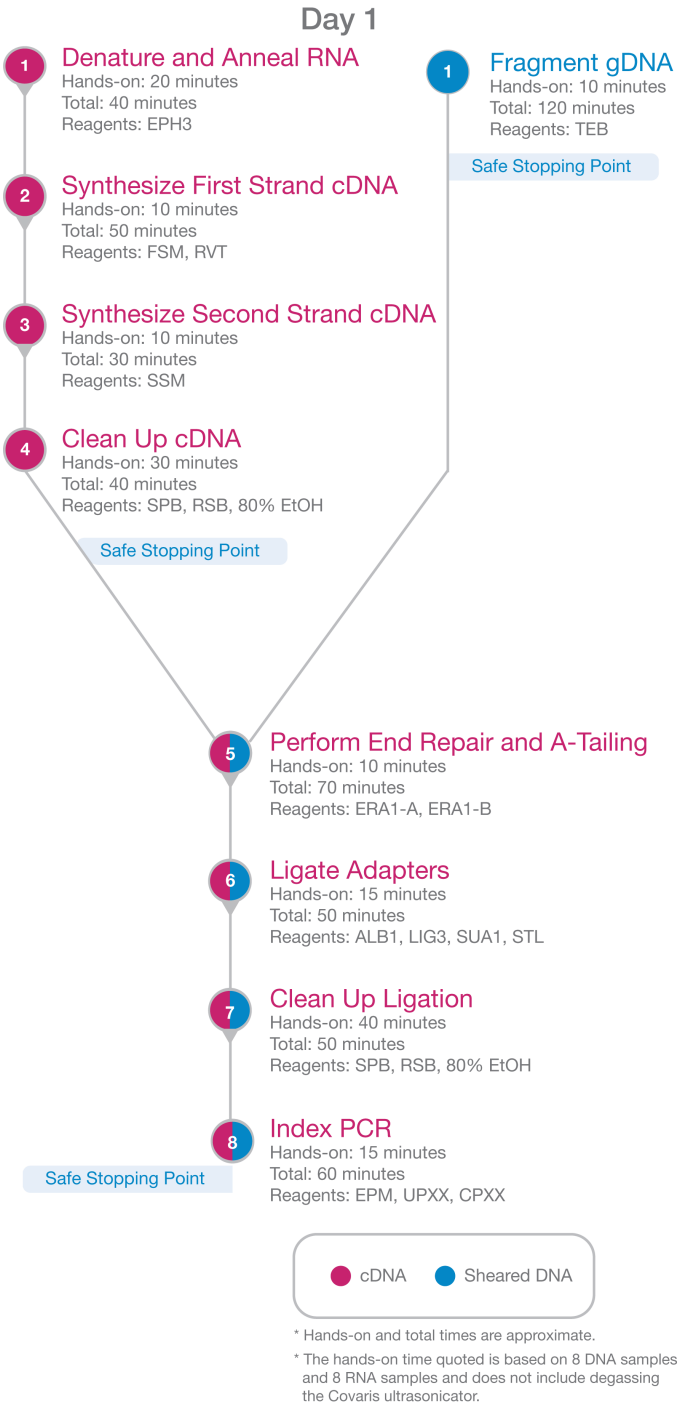
- Adjust the volume setting to ~50–75% of your sample volume.
- Pipette with a slow, smooth action without releasing the plunger.
- Avoid aggressive pipetting, splashing, and introducing bubbles.
- Position the pipette tip above the pellet and dispense directly into the pellet to release beads from the well or tube.
- Make sure 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 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 plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Dispense liquid 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 Workflow

The following diagram illustrates the recommended library preparation workflow using a TruSight Tumor 170 kit. RNA and DNA libraries can be prepared simultaneously. Safe stopping points are marked between steps.

Figure 1 TruSight Tumor 170 Workflow (Part 1)



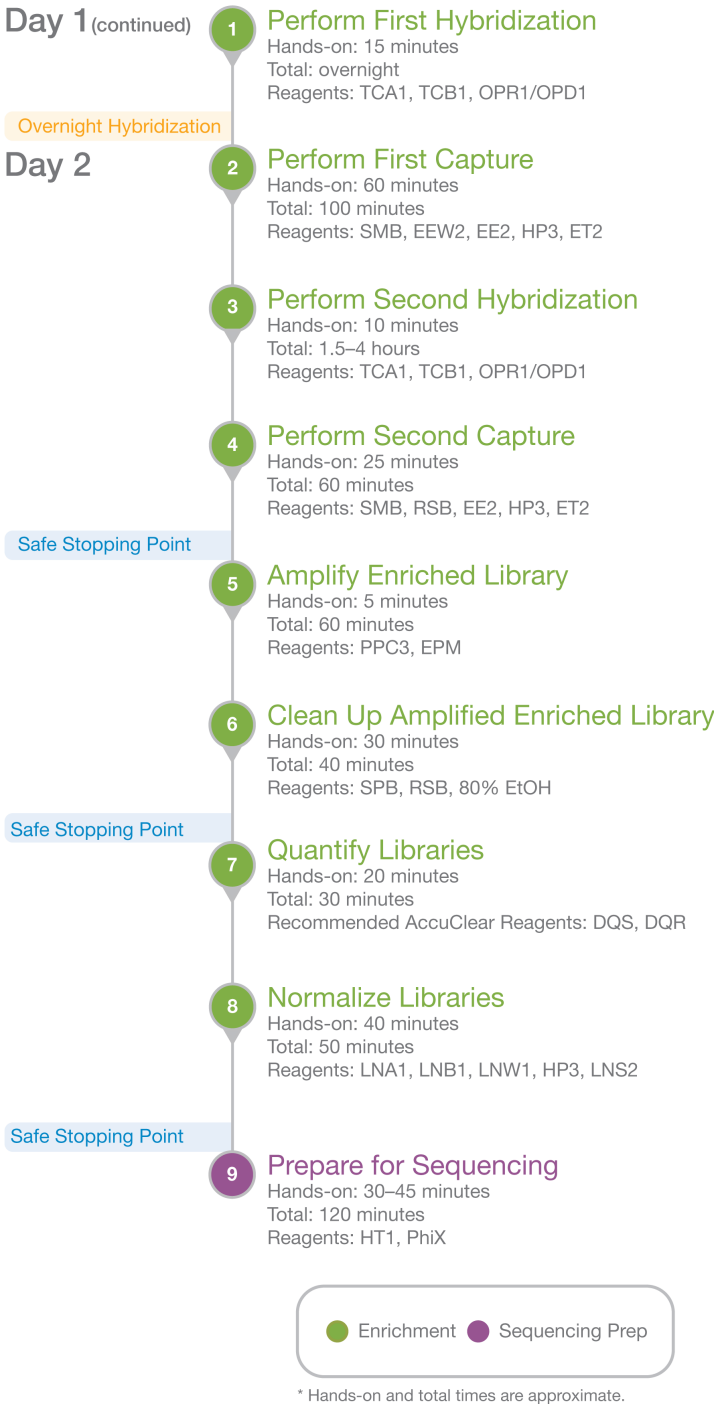
* Hands-on and total times are approximate.

* The hands-on time quoted is based on 8 DNA samples and 8 RNA samples and does not include degassing the Covaris ultrasonicator.

Enrichment Workflow

The following diagram illustrates the recommended enrichment workflow using a TruSight Tumor 170 kit. Safe stopping points are marked between steps.

Figure 2 TruSight Tumor 170 Workflow (Part 2)



* Hands-on and total times are approximate.

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 12.

Consumables

- Nuclease-free water
- EPH3 (Elute, Prime, Fragment High Mix 3 [red cap])
- FSM (First Strand Synthesis Mix [red cap])
- RVT (Reverse Transcriptase [red cap])
- 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.

Item	Storage	Instructions
EPH3	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
FSM	-25°C to -15°C	Thaw and bring 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 *RNA/DNA Input Recommendations* on page 1.

4 Dilute each purified RNA sample to a concentration between 4.7 ng/µl and 10 ng/µl in RNase/DNase-free water.
   - Use a minimum of 40 ng and a maximum of 120 ng for each purified DNA sample.

5 Save the following programs on a thermal cycler:
   - For FFPE or fragmented RNA, save the LQ-RNA program.
     - Choose the preheated 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 preheated 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 reagents in a microcentrifuge tube to create FSM+RVT master mix.

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

- 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 9.

4 Add 8.5 µl of each purified RNA sample (4.7 ng/µl to 10 ng/µl) to the corresponding well of the CF 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.

8 When the thermal cycler reaches 4°C, proceed immediately to *Synthesize First Strand cDNA* on page 9.

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 (prepared in *Denature and Anneal RNA* on page 8)
- Microseal 'B' adhesive seals

Preparation

1 Save the following program as 1stSS on a thermal cycler with a heated lid:
- Choose the preheated 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 plate from the thermal cycler.

2 Pipette to mix FSM+RVT master mix before use.

3 Add 8 µl FSM+RVT master mix to each well.

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

5 Place on a thermal cycler and run the 1stSS program.

6 When the thermal cycler reaches 4°C, proceed immediately to *Synthesize Second Strand cDNA* on page 10. 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 12 to begin.

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.

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

2 Save the following program as 2ndSS on a 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 preheated 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 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 a thermal cycler and run the 2ndSS program.

5 When the thermal cycler reaches 4°C, proceed to *Clean Up cDNA* on page 10.

Clean Up cDNA

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

Consumables

- Freshly prepared 80% ethanol (EtOH)
- SPB (Sample Purification Beads)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive seals
- 96-well midi plates (1–2)

- [Optional] 96-well PCR plate

CAUTION

Proper plate type is required for optimal assay performance. To continue the protocol after this step, use a midi plate. To store the samples after this step, use a PCR plate. See *Preparation* on page 11 for more information.

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- 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 at least 30 minutes. Before using SPB, vortex for 1 minute.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If RSB was stored at -25°C to -15°C, thaw at room temperature and vortex before use.

2 Label a new 96-well midi plate BIND1.

3 Label a new 96-well midi plate PCF (Purified cDNA Fragments).
- [Optional] To store the plate after this step, use a new 96-well PCR plate.

4 Prepare fresh 80% EtOH.

Procedure

Bind

1 Remove the CF plate from the thermal cycler.

2 Add 90 µl SPB to each well of the BIND1 midi plate.

3 Transfer 50 µl of each sample from the CF plate to the corresponding well of the BIND1 midi plate.

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

5 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 as follows:

a While on the magnetic stand, add 200 µl fresh 80% EtOH.

b Wait 30 seconds, then remove and discard all supernatant from each well.

4 Repeat step 3 (a–b) to wash a second time.

NOTE

Two washes are required for optimal assay performance.

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 of 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 to mix (a minimum of 10 times).

8 Proceed to *Perform End Repair and A-Tailing* on page 14 or apply Microseal 'B' and store. If you are also preparing DNA libraries, 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* on page 12 for more information.

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 optimally fragments gDNA to a 90–250 bp fragment size using the Covaris Focused-ultrasonicator. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs.

If you are working with only purified RNA, skip this step and proceed directly to *Perform End Repair and A-Tailing* on page 14.

If you are also preparing cDNA libraries from RNA samples, purified cDNA fragments and sheared DNA samples can be stored separately in the PCF plate. Make sure to label wells. See *Preparation* on page 13 for more information.

Consumables

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

CAUTION

Proper plate type is required for optimal assay performance. To continue the protocol after this step, use a midi plate. To store the samples after this step, use a PCR plate. See *Preparation* on page 13 for more information.

Preparation

1 Prepare the following consumables.

Item	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.

3 Choose 1 of the following plate options:
- To process only gDNA, use a new 96-well midi plate.
- To process cDNA samples simultaneously, continue to use the PCF plate from *Clean Up cDNA* on page 10.
- [Optional] To store sheared gDNA after this step, use a 96-well PCR plate.

4 Label the plate LP (Library Preparation).

5 Thaw gDNA samples at room temperature. Invert to mix.

6 See *RNA/DNA Input Recommendations* on page 1 to qualify or quantify samples.

7 Dilute each purified DNA sample to a concentration between 3.3 ng/µl and 10 ng/µl in TEB.
- Use a minimum of 40 ng and a maximum of 120 ng for each purified DNA sample.
- The final volume must be at least 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 [Optional] If you are using the LE220 Covaris instrument, fill the unused Covaris 8 microTUBE Strip wells with 52 µl of water for optimal performance.

4 Pipette to mix.

5 Seal the microTUBE Strip with the foil seal.

6 Centrifuge briefly.

7 If you are using the Covaris E220 *evolution* or LE220 model, fragment the gDNA using the following settings.
If you are using a different Covaris model, consult the TruSight Tumor 170 support page.

Setting	E220 *evolution*	LE220
Peak Incident Power	175 watts	450 watts
Duty Factor	10%	30%
Cycles per Burst	200	200
Treatment Time	280 seconds	250 seconds
Temperature	7°C	7°C
Intensifier	yes	N/A

8 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).

9 [Optional] If the PCF plate is a midi plate and you plan to store it after this step, transfer 50 µl of cDNA and 50 µl of sheared gDNA sample to the corresponding wells of a new 96-well PCR plate.
- 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).
- Label the plate LP.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PCR 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 overhangs resulting from fragmentation 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)
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tube
- [Optional] 96-well midi plate

CAUTION

If a PCR plate was used to store the gDNA and/or cDNA samples, follow the plate transfer instructions in step 4 of the *Preparation* on page 14.

Preparation

1 Prepare the following consumables.

Item	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 and bring to room temperature. Centrifuge briefly, and then pipette to mix. If crystals are present, warm the tube in your hands, and then pipette to mix until the crystals are dissolved.

2 Bring cDNA and sheared gDNA to room temperature.

3 If cDNA and gDNA samples are stored in separate midi plates, move all samples to the same midi plate.

4 [Optional] If cDNA and/or sheared gDNA samples are stored in a 96-well PCR plate(s), transfer 50 µl of cDNA and/or sheared gDNA sample to the corresponding wells of a new, single 96-well midi plate.

5 Label (or relabel) the midi plate LP2 (Library Preparation 2).

6 Preheat 2 Hybex incubators with midi heat block inserts as follows:
- Preheat a Hybex incubator to 30°C.
- Preheat a Hybex incubator to 72°C.

Procedure

1 Combine the following reagents in a microcentrifuge tube to create ERA1 master mix.

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

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

2 Pipette to mix (a minimum of 10 times) and place ERA1 master mix on ice.

3 Add 10 µl ERA1 master mix to each sample in the LP2 plate.

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

5 Incubate in a Hybex incubator at 30°C for 30 minutes.

6 Immediately transfer to another Hybex 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.

Consumables

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

About Reagents

- ALB1 is highly viscous. Pipette slowly to avoid forming bubbles.

Preparation

1 Prepare the following consumables.

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

Procedure

1 Add 60 µl ALB1 to each well.

2 Add 5 µl LIG3 to each well.

3 Vortex SUA1 a minimum of 10 seconds.

4 Add 10 µl SUA1 to each well.

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

6 Incubate at room temperature for 30 minutes.

7 Add 5 µl STL.

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

Clean Up Ligation

This process uses SPB to purify the cDNA or gDNA fragments and remove unwanted products.

Consumables

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

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the suspension.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
SPB	2°C to 8°C	Bring to room temperature. Before using SPB, vortex for 1 minute.
RSB	2°C to 8°C -25°C to -15°C	Bring to room temperature. If RSB was stored at -25°C to -15°C, thaw at room temperature and vortex before use.

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

3 Prepare fresh 80% EtOH.

Procedure

Bind

1 Add 112 µl SPB to each well in the LP2 plate.

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

3 Incubate at room temperature for 5 minutes.

Wash

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

2 Remove and discard all supernatant from each well.

3 Wash as follows:

   a While on the magnetic stand, add 200 µl fresh 80% EtOH.
   b Wait 30 seconds, then remove and discard all supernatant from each well.

4 Repeat step 3 (a–b) to wash a second time.

> **NOTE**
> Two washes are required for optimal assay performance.

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 1500 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 plate to the corresponding well of the LS plate.

Index PCR

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

> **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.

Consumables

- EPM (Enhanced PCR Mix)
- UPXX (Unique Index Primer Mixes), see *Kit Contents* on page 33
- CPXX (Combinatorial Index Primer Mixes), see *Kit Contents* on page 33
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
UPXX	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
CPXX	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.

2 Assign 1 UPXX index primer mix per RNA library and 1 CPXX index primer mix per DNA library (XX = index primer mix number).

- UPXX index primer mixes can also be used with DNA libraries.
- Do not use CPXX index primer mixes with RNA libraries.
- Low-plex sequencing runs should use 3 libraries containing 1 of the following UPXX index primer sets to provide sufficient diversity.

  Choose 1 of the following index primer sets for low-plex runs:
  - [UP01,UP02,UP03]
  - [UP04,UP05,UP06]
  - [UP07,UP08,UP09]
  - [UP10,UP11,UP12]

For more information, see *Kit Contents* on page 33.

CAUTION

If you are sequencing multiple libraries on a single flow cell, assign a different indexing primer mix to each library sample.

CAUTION

When handling indexing primers, avoid cross-contamination. After an indexing primer mix tube is opened, discard the original tube cap and apply a new tube cap.

CAUTION

Make sure to assign UPXX index primer mixes to RNA libraries only. Assigning CPXX index primer mixes to RNA libraries may result in decreased performance.

3 In the post-amp area, save the following program as I-PCR on a thermal cycler with a heated lid:

- Choose the preheated 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 of indexing primer mix (UPXX or CPXX) to each well in the LS plate.
(See *Preparation* on page 18 for more information about indexing primer mixes.)

2 Add 20 µl EPM to each well.

3 Apply Microseal 'B' and shake the plate at 1500 rpm 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 After the I-PCR program completes, relabel the plate ALS (Amplified Library Samples).

7 Centrifuge briefly.

SAFE STOPPING POINT

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

Perform First Hybridization

During this process, a pool of oligos specific to the 170 genes targeted by TruSight Tumor 170 is hybridized to the RNA and/or DNA libraries generated during *Index PCR* on page 17. Two hybridization steps are required to ensure enrichment of the targeted regions. In this step, the first hybridization is performed overnight (8 hours to 24 hours).

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.

Consumables

- TCA1 (Target Capture Additives 1)
- TCB1 (Target Capture Buffer 1)
- OPR1 (Oncology Probes RNA 1 [red cap])
- OPD1 (Oncology Probes DNA 1 [blue cap])
- 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Use OPR1 for RNA libraries only (red cap).
- Use OPD1 for DNA libraries only (blue cap).

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
TCB1	2°C to 8°C	Bring to room temperature. Vortex for 1 minute to resuspend. Centrifuge briefly. If crystals are present, warm the tube in your hands, and then vortex until the crystals are dissolved.
TCA1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
OPD1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.

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

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

4 Save the following program as HYB1 on a thermal cycler with a heated lid:
- Choose the preheated 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 RNA and/or DNA library to the HYB1 plate.

2 Add 15 µl TCB1 to each well.

3 Add 10 µl TCA1 to each well.

4 Add the appropriate probe:
- For RNA libraries, add 5 µl of OPR1 (red cap).
- For DNA libraries, add 5 µl of OPD1 (blue cap).

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

6 Place on the preprogrammed thermal cycler and run the HYB1 program. Hybridize at 57°C overnight (minimum of 8 hours to a maximum of 24 hours).

Perform First Capture

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Three heated washes using EEW2 remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

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.

Consumables

- SMB (Streptavidin Magnetic Beads)
- ET2 (Elute Target Buffer 2)
- EE2 (Enrichment Elution 2)
- HP3 (2 N NaOH)
- EEW2 (Enhanced Enrichment Wash 2)
- 96-well midi plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.
- Vortex SMB frequently to make sure that beads are suspended.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE2	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
EEW2	-25°C to -15°C	Thaw and bring to room temperature. Vortex for 1 minute to resuspend.
SMB	2°C to 8°C	Bring to room temperature. Vortex for 1 minute. If the bead pellet is present, pipette up and down to release the pellet, and then vortex 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.

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

3 Label a new 96-well midi plate CAP1.

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

Procedure

Bind

1 Remove the HYB1 plate from the thermal cycler.

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

3 Transfer 50 µl of each library from the HYB1 plate to the corresponding well in the CAP1 plate.

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

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

6 Place on a magnetic stand for 2 minutes.

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

Wash

1 Wash as follows:

   a Remove the CAP1 plate from the magnetic stand.
   b Add 200 µl EEW2 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 Repeat step 1 (a–g) to wash a second time.

3 Repeat step 1 (a–g) to wash a third time.

NOTE

Three washes are required for optimal assay performance.

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

Elute

1 Combine the following reagents in a microcentrifuge tube to create EE2+HP3 Elution Mix.

Elution Mix Component	Per 3 Samples	Per 8 Samples	Per 16 Samples	Per 24 Samples
EE2	95 µl	228 µl	456 µl	684 µl
HP3	5 µl	12 µl	24 µl	36 µl

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

2 Vortex briefly to mix.

3 Remove the CAP1 plate from the magnetic stand.

4 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 of eluate from each well of the CAP1 plate into the ELU1 plate.

CAUTION

Transferring less than the specified volume of eluate may affect assay performance.

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

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

Perform 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, the second hybridization step should be performed for a minimum of 1.5 hours to a maximum of 4 hours.

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.

Consumables

- TCA1 (Target Capture Additives 1)
- TCB1 (Target Capture Buffer 1)
- OPR1 (Oncology Probes RNA 1 [red cap])
- OPD1 (Oncology Probes DNA 1 [blue cap])
- Microseal 'B' adhesive seals

About Reagents

- Use OPR1 for RNA libraries only (red cap).
- Use OPD1 for DNA libraries only (blue cap).

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
TCB1	2°C to 8°C	Bring to room temperature. Vortex for 1 minute to resuspend. Centrifuge briefly. If crystals are present, warm the tube in your hands, and then vortex until the crystals are dissolved.
TCA1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
OPD1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.

2 Save the following program as HYB2 on a thermal cycler with a heated lid:
- Choose the preheated 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 in the ELU1 plate.

2 Add 10 µl TCA1 to each well.

3 Add the appropriate probe:
- For RNA libraries, add 5 µl OPR1 (red cap).
- For DNA libraries, add 5 µl OPD1 (blue cap).

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

5 Place on the preprogrammed thermal cycler and run the HYB2 program. Hybridize at 57°C for a minimum of 1.5 hours to a maximum of 4 hours.

Perform Second Capture

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.

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.

Consumables

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

About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.
- Vortex SMB frequently to make sure that beads are suspended.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE2	-25C to -15C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
SMB	2°C to 8°C	Bring to room temperature. Vortex for 1 minute. If the bead pellet is present, pipette up and down to release the pellet, and then vortex 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.
RSB	2°C to 8°C -25°C to -15°C	Bring to room temperature. If RSB was stored at -25°C to -15°C, thaw at room temperature and vortex before use.

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

3 Label a new 96-well midi plate CAP2 .

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

Procedure

Bind

1 Remove the ELU1 plate from the thermal cycler.

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

3 Transfer 50 µl of each library from the ELU1 plate to the corresponding well of the CAP2 plate.

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

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

6 Place on a magnetic stand for 2 minutes.

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

Wash

1 Remove the CAP2 plate from the magnetic stand.

2 Add 200 µl of RSB to each well.

3 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'.

4 Place on a magnetic stand for 2 minutes.

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

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

Elute

1 Combine the following reagents in a microcentrifuge tube to create a fresh EE2+HP3 Elution Mix.

Elution Mix Component	Per 3 Samples	Per 8 Samples	Per 16 Samples	Per 24 Samples
EE2	95 µl	228 µl	456 µl	684 µl
HP3	5 µl	12 µl	24 µl	36 µl

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

2 Vortex to mix.

3 Remove the CAP2 plate from the magnetic stand.

4 Add 22 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 Transfer 20 µl of eluate from each well of the CAP2 plate into the ELU2 plate.

CAUTION

Transferring less than the specified volume of eluate may affect assay performance.

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

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

SAFE STOPPING POINT

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

Amplify Enriched Library

This step uses primers to amplify enriched libraries.

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.

Consumables

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

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Vortex to resuspend. Centrifuge briefly.
PPC3	-25°C to -15°C	Thaw and bring 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 and centrifuge.

3 Save the following program as EL-PCR on a thermal cycler with a heated lid:
- Choose the preheated 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 plate.

2 Add 20 µl EPM to each well.

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

4 Briefly centrifuge at 280 × g.

5 Place on a 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

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

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.

- 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 . Before using SPB, vortex for 1 minute.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If RSB was stored at -25°C to -15°C, thaw at 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 plate from the thermal cycler.

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

3 Transfer 50 µl of each library from the ELU2 plate to the corresponding well of the BIND2 plate.

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

5 Incubate at room temperature for 5 minutes.

Wash

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

2 Remove and discard all supernatant from each well.

3 Wash as follows:

a While on the magnetic stand, add 200 µl fresh 80% EtOH.
b Wait 30 seconds, then remove and discard all supernatant from each well.

4 Repeat step 3 (a–b) to wash a second time.

NOTE
Two washes are required for optimal assay performance.

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

Elute

1 Remove the BIND2 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 plate to the corresponding well of the PL plate.

SAFE STOPPING POINT

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

Quantify Libraries

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

1 The DNA standard provided with the fluorometric quantification kit, the libraries, and the blank solution should all be run in triplicate.

2 Determine the average relative fluorescence unit (RFU) for each.

3 Calculate the following values:
- Average Standard RFU - Average Blank RFU = Normalized Standard RFU
- Average 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 or RNA 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 may not yield adequate sequencing results needed to confidently call variants that may 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.

Manual library normalization is not currently supported by TruSight Tumor 170. Contact Illumina Technical Support if you would like to manually normalize libraries.

Before proceeding, see the documentation for your sequencing system to make sure that normalization methods are compatible. Do not use a bead-based normalization method on libraries being sequenced on a system with onboard denaturation.

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.

Consumables

- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNW1 (Library Normalization Wash 1)
- LNS2 (Library Normalization Storage 2)
- HP3 (2 N NaOH)
- PCR-grade water
- 96-well PCR plate
- 96-well midi plate
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tubes (2)

About Reagents

- Vortex LNB1 for 1 minute before each use.
- Vortex LNB1 frequently to make sure that beads are evenly distributed.
- Aspirate and dispense LNB1 slowly due to the viscosity of the suspension.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
LNA1	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
LNS2	15°C to 30°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
LNB1	2°C to 8°C	Bring to room temperature. Vortex for 1 minute to make sure that beads are evenly distributed. Pipette LNB1 pellet up and down to ensure resuspension.
LNW1	2°C to 8°C	Bring to room temperature. Vortex to resuspend.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.

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

3 Label a new 96-well midi plate BIND3.

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

Procedure

1 Combine the following reagents in a new microcentrifuge tube to create LNA1+LNB1 master mix.

Master Mix Component	Per 3 Samples	Per 8 Samples	Per 16 Samples	Per 24 Samples
LNA1	132 µl	352 µl	704 µl	1056 µl
LNB1	24 µl	64 µl	128 µl	192 µl

2 Vortex to mix.

3 Combine the following reagents in a new microcentrifuge tube to create a fresh 0.1 N NaOH solution.

Solution Component	Per 3 Samples	Per 8 Samples	Per 16 Samples	Per 24 Samples
PCR-grade water	114 µl	304 µl	608 µl	912 µl
HP3	6 µl	16 µl	32 µl	48 µl

4 Vortex to mix.

Bind

1 Add 45 µl LNA1+LNB1 master mix to each well of the BIND3 plate.

2 Add 20 µl of each library from the PL plate to the corresponding well of the BIND3 plate.

3 Apply Microseal 'B' and shake at 1800 rpm for 10 minutes.

4 Place the BIND3 plate on a magnetic stand for 2 minutes.

5 Remove and discard all supernatant from each well.

Wash

1 Wash as follows:

   a Remove the BIND3 plate from the magnetic stand.
   b Add 45 µl of LNW1 to each well.
   c Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.
   d Place on a magnetic stand for 2 minutes.
   e Remove and discard all supernatant from each well.

2 Repeat step 1 (a–e) to wash a second time.

NOTE

Two washes are required for optimal assay performance.

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

Elute

1 Add 32 µl 0.1 N NaOH solution to each well.

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

3 Place BIND3 on a magnetic stand for 2 minutes.

4 Transfer 30 µl of each eluate from the BIND3 plate to the corresponding well of the NL plate.

5 Add 30 µl LNS2 to each library in the NL plate.

6 Pipette up and down to mix.

SAFE STOPPING POINT

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

Pool Libraries

Use the following protocol to pool libraries.

Consumables

- Microcentrifuge tubes (screw top)

Preparation

1. If the NL plate was stored at -25°C to -15°C, thaw at room temperature and centrifuge.
2. Label a screw top microcentrifuge tube PRL (Pooled RNA Libraries).
3. Label a screw top microcentrifuge tube PDL (Pooled DNA Libraries).
4. To prepare for the sequencing run, begin thawing reagents according to the instructions for your sequencing instrument.

Procedure

1. Transfer 10 µl of each normalized RNA library from the NL plate to the PRL tube.
2. Transfer 10 µl of each normalized DNA library from the NL plate to the PDL tube.
3. Vortex each tube to mix.
4. Centrifuge each tube briefly.

Dilute Libraries to the Loading Concentration

Denature and dilute libraries to the starting concentration for your sequencing system. After denaturing and diluting to the starting concentration, libraries are ready to be diluted to the final loading concentration.

For sequencing, Illumina recommends setting up a paired-end run with 101 cycles per read (2 × 101) and 10 cycles per index read.

1. Follow the denature and dilute libraries guide for your system to dilute to the final loading concentration. The final loading concentrations are a starting point and general guideline. If necessary, optimize dilutions for your workflow over subsequent sequencing runs or by flow cell titration.

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.

Acronyms

Acronym	Definition
1stSS	1st Strand Synthesis
2ndSS	2nd Strand Synthesis
ALS	Amplified Library Samples
cDNA	Complementary DNA
CF	cDNA Fragments
ELU1	Elution 1
ELU2	Elution 2
gDNA	Genomic DNA
HQ-RNA	High-quality RNA
HYB1	Hybridization 1
LQ-RNA	Low-quality RNA
LS	Library Samples
LP	Library Preparation
LP2	Library Preparation 2
NL	Normalized Libraries
PCF	Purified cDNA Fragments
PDL	Pooled DNA Libraries
PL	Purified Libraries
PRL	Pooled RNA Libraries

Kit Contents

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

NOTE

The reagents in box numbers 1, 8, and 9 have color-coded caps to identify the associated library.

- Blue caps identify reagents used only with DNA libraries.
- Red caps identify reagents used only with RNA libraries.

Consumable	Catalog #
TruSight Tumor 170 NextSeq Kit (24 Sample Library Prep Kit	20028821
TruSight Tumor 170 (1 Library Prep, 24 Samples)	20028822

Library Prep

Box 1 - Library Prep – RNA (Pre-Amp)

Quantity	Reagent	Description	Storage Temperature
1	FSM	First Strand Synthesis Mix	-25°C to -15°C
1	SSM	Second Strand Mix	-25°C to -15°C
1	EPH3	Elute, Prime, Fragment High Mix 3	-25°C to -15°C
1	RVT	Reverse Transcriptase	-25°C to -15°C

Box 2 - Library Prep – DNA (Pre-Amp)

Quantity	Reagent	Description	Storage Temperature
2	ERA1-A	End Repair A-tailing Enzyme Mix 1	-25°C to -15°C
2	ERA1-B	End Repair A-tailing Buffer 1	-25°C to -15°C
2	ALB1	Adapter Ligation Buffer 1	-25°C to -15°C
2	LIG3	DNA Ligase 3	-25°C to -15°C
2	SUA1	Short Universal Adapters 1	-25°C to -15°C
2	STL	Stop Ligation Buffer	-25°C to -15°C
2	EPM	Enhanced PCR Mix	-25°C to -15°C

Box 3 - Library Prep (Pre-Amp)

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 4 - Library Prep - Unique PCR Index Primer Mixes (Pre-Amp)

Quantity	Reagent	Description	Storage Temperature
1	UP01	Unique Index Primer Mix 01	-25°C to -15°C
1	UP02	Unique Index Primer Mix 02	-25°C to -15°C
1	UP03	Unique Index Primer Mix 03	-25°C to -15°C
1	UP04	Unique Index Primer Mix 04	-25°C to -15°C
1	UP05	Unique Index Primer Mix 05	-25°C to -15°C
1	UP06	Unique Index Primer Mix 06	-25°C to -15°C
1	UP07	Unique Index Primer Mix 07	-25°C to -15°C
1	UP08	Unique Index Primer Mix 08	-25°C to -15°C
1	UP09	Unique Index Primer Mix 09	-25°C to -15°C
1	UP10	Unique Index Primer Mix 10	-25°C to -15°C
1	UP11	Unique Index Primer Mix 11	-25°C to -15°C
1	UP12	Unique Index Primer Mix 12	-25°C to -15°C
1	UP13	Unique Index Primer Mix 13	-25°C to -15°C
1	UP14	Unique Index Primer Mix 14	-25°C to -15°C
1	UP15	Unique Index Primer Mix 15	-25°C to -15°C
1	UP16	Unique Index Primer Mix 16	-25°C to -15°C

Box 5 - Library Prep – Combinatorial PCR Index Primer Mixes (Pre-Amp)

Quantity	Reagent	Description	Storage Temperature
1	CP01	Combinatorial Index Primer Mix 01	-25°C to -15°C
1	CP02	Combinatorial Index Primer Mix 02	-25°C to -15°C
1	CP03	Combinatorial Index Primer Mix 03	-25°C to -15°C
1	CP04	Combinatorial Index Primer Mix 04	-25°C to -15°C
1	CP05	Combinatorial Index Primer Mix 05	-25°C to -15°C
1	CP06	Combinatorial Index Primer Mix 06	-25°C to -15°C
1	CP07	Combinatorial Index Primer Mix 07	-25°C to -15°C
1	CP08	Combinatorial Index Primer Mix 08	-25°C to -15°C
1	CP09	Combinatorial Index Primer Mix 09	-25°C to -15°C
1	CP010	Combinatorial Index Primer Mix 10	-25°C to -15°C
1	CP011	Combinatorial Index Primer Mix 11	-25°C to -15°C
1	CP012	Combinatorial Index Primer Mix 12	-25°C to -15°C
1	CP013	Combinatorial Index Primer Mix 13	-25°C to -15°C
1	CP014	Combinatorial Index Primer Mix 14	-25°C to -15°C
1	CP015	Combinatorial Index Primer Mix 15	-25°C to -15°C
1	CP016	Combinatorial Index Primer Mix 16	-25°C to -15°C

Enrichment

Box 6 - Enrichment (Post-Amp)

Quantity	Reagent	Description	Storage Temperature
2	TCB1	Target Capture Buffer 1	2°C to 8°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
2	HP3	2 N NaOH	2°C to 8°C
2	ET2	EluteTarget Buffer 2	2°C to 8°C
1	LNB1	Library Normalization Beads 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	SPB	Sample Purification Beads	2°C to 8°C
1	LNS2	Library Normalization Storage 1	15°C to 30°C

- Remove the LNS2 tubes from the 2°C to 8°C Box and store at 15°C to 30°C upon receipt.

Box 7 - Enrichment (Post-Amp)

Quantity	Reagent	Description	Storage Temperature
2	TCA1	Target Capture Additives 1	-25°C to -15°C
1	EEW2	Enhanced Enrichment Wash	-25°C to -15°C
2	EE2	Enrichment Elution 2	-25°C to -15°C
2	EPM	Enhanced PCR Mix	-25°C to -15°C
2	PPC3	PCR Primer Cocktail 3	-25°C to -15°C
2	LNA1	Library Normalization Additives 1	-25°C to -15°C

Box 8 - TruSight Tumor 170 Content Set (DNA Only)

Quantity	Reagent	Description	Storage Temperature
1	OPD1	Oncology DNA Probes Master Pool	-25°C to -15°C

Box 9 - TruSight Tumor 170 Content Set (RNA Only)

Quantity	Reagent	Description	Storage Temperature
1	OPR1	Oncology RNA Probes Master Pool	-25°C to -15°C

Consumables and Equipment

Confirm that all required user-supplied consumables and equipment are present and available 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	Advanced Analytical Technologies, 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	Covaris, part # 520053
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
1.7 ml microcentrifuge tubes, nuclease-free	General lab supplier
2 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
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
Nuclease-free water	General lab supplier
RNase/DNase-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 (115V) or • 1057-30-2 (230V)

Equipment	Supplier
(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*)
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Fragment Analyzer Automated CE System	Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10

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 (115V) or • 1057-30-2 (230V)
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	Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

Document # 1000000024091 v02

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