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TruSight Oncology 500 ctDNA

Reference Guide

ILLUMINA PROPRIETARY
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Revision History

Document	Date	Description of Change
Document # 1000000092559 v01	August 2025	Clarified SMB thaw and use instructions.
Document # 1000000092559 v00	February 2020	Initial release.

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Overview

Introduction

The TruSight™ Oncology 500 Circulating Tumor DNA (ctDNA) protocol describes an enrichment-based approach to convert cell-free DNA (cfDNA) extracted from plasma samples into libraries enriched for cancer-related genes that can be sequenced on Illumina® sequencing systems. The TruSight Oncology 500 ctDNA Kit enables the preparation of 48 libraries from cfDNA.

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

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

TruSight Oncology 500 ctDNA also detects immunotherapy biomarkers for tumor mutational burden (TMB) and microsatellite instability (MSI).

cfDNA Input Recommendations

- The TruSight Oncology 500 ctDNA Kit assay requires a minimum of 30 ng cfDNA input.
- Quantify the nucleic acids before starting the assay.
- To quantify the nucleic acids, use a capillary electrophoresis-based method, such as the Agilent Fragment Analyzer.
- To ensure optimal nucleic acid input, quantify the cfDNA fraction only.
- For recommendations for obtaining sufficient nucleic acid material, see the TruSight Oncology 500 ctDNA support page on the Illumina Support Center.

Compatibility

For information on sequencing compatibility and run settings, see the TruSight Oncology 500 ctDNA support pages on the Illumina Support Center.

For read lengths, see the compatible products page on the TruSight Oncology 500 support page on the Illumina Support Center.

Reference Samples

- Use reference materials with known variant composition, such as SeraSeq ctDNA Complete Mutation Mix.
- Use RNase/DNase-free water as a non-template control. Do not sequence the non-template control.
- Processing a reference sample or non-template control reduces the total number of test samples that can be processed.

Additional Resources

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

Resource	Description
TruSight Oncology 500 ctDNA Checklist (document # 1000000107605)	Provides a checklist of steps for the experienced user.
TruSight Oncology 500 ctDNA Consumables & Equipment List (document # 1000000107604)	Provides an interactive checklist of user-provided consumables and equipment.
Illumina Adapter Sequences (document # 1000000002694)	Provides adapter sequences for Illumina library prep kits.

Protocol

This section describes the TruSight Oncology 500 ctDNA 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.
- Follow the protocol in the order described using the specified parameters.
- Before beginning library preparation, record sample information, and assign each sample a unique index.

Prepare for Pooling

Record information about your samples before starting library prep. For compatibility information, see the TruSight Oncology 500 ctDNA support pages on the Illumina Support Center or the support pages for your system.

- For index adapter sequences and how to record them, see *Illumina Adapter Sequences (document # 1000000002694)*.

Tips and Techniques

Protocol Continuity

- Review tips and techniques before starting the protocol, as many critical techniques are listed only here and are not repeated in the protocol.
- Follow the protocol in the order described using the specified parameters.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

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

- Clean work surfaces and equipment thoroughly before and after the procedure with an RNase/DNase inhibiting cleaner.
- Handle and open only one index primer at a time. Recap each index tube immediately after use. Extra caps are provided with the kit.

Sealing the Plate

- Always seal the plate 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.
- Use Microseal 'B' adhesive seals for shaking, centrifuging, and long-term storage. The seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates.
- If you observe droplets hanging from the inside of a sealed plate, centrifuge at 280 x 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 x 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.

Handling Beads

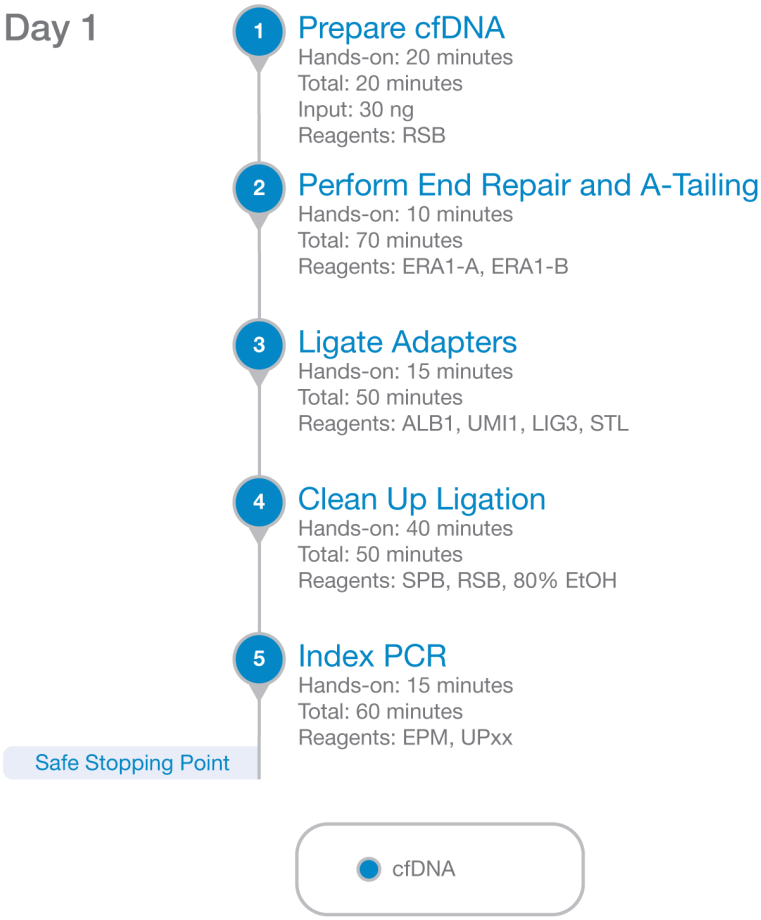
- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).

- When washing beads:
  - Use the specified 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 it is on the magnetic stand. Do not disturb the bead pellet.
- 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.
  - Mix beads for 1 minute to ensure homogeneity.
  - 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.)

Library Prep Workflow

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

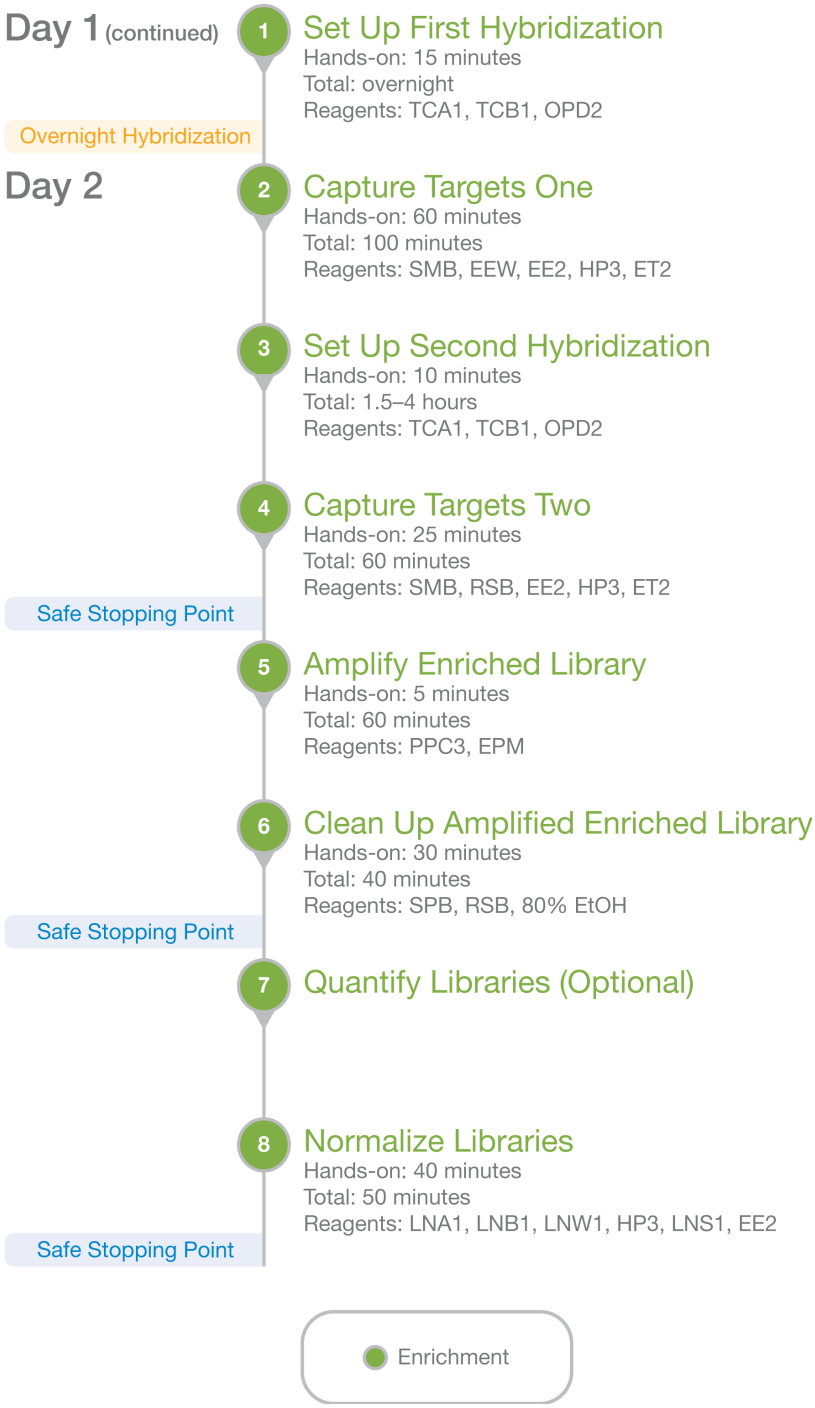
Figure 1 TruSight Oncology 500 ctDNA Kit Workflow (Part 1)



Enrichment Workflow

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

Figure 2 TruSight Oncology 500 ctDNA Kit Workflow (Part 2)



Prepare cfDNA and Perform End Repair and A-Tailing

This process converts the 5' and 3' overhangs on cfDNA 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)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tube
- 96-well MIDI plate

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, and then pipette to mix. If precipitates are present, warm the tube in your hands, and then pipette to mix until the crystals dissolve.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. Vortex before use. If stored at -25°C to -15°C, thaw at room temperature and vortex before use.

2. Bring cfDNA to room temperature.
3. See *cfDNA Input Recommendations* on page 1 to quantify samples.
4. Use RSB to prepare a minimum of 30 ng of each purified cfDNA sample in a final volume of 50 µl (0.6 ng/µl).
5. Pipette mix or vortex cfDNA and then centrifuge briefly.
6. Label the MIDI plate LP (Library Preparation).
7. 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.
8. Transfer 50 µl of each cfDNA sample to corresponding wells of a new 96-well MIDI plate.
9. Prepare an ice bucket.

Procedure

1. Combine the appropriate volumes from the table below in a microcentrifuge tube to prepare ERA1 Master Mix. Please note that master mix volumes include overage.

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 for a minimum of 3 libraries.
- 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 LP MIDI plate.
4. Apply Microseal 'B' to the LP MIDI plate and shake the plate at 1800 rpm for 1 minute.
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 LP MIDI plate on ice for 5 minutes.

Ligate Adapters

UMI1 adapters that contain unique molecular indexes are ligated to cfDNA fragments. This process ligates adapters to the ends of cfDNA fragments.

Consumables

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

About Reagents

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

Preparation

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.

Item	Storage	Instructions
STL	-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 10 µl UMI1 to each well.
4. Apply Microseal 'B' to the LP MIDI plate and shake the plate at 1800 rpm for 1 minute.
5. Incubate at room temperature for 30 minutes.
6. Add 5 µl STL to each well.
7. Apply Microseal 'B' to the LP MIDI plate and shake the plate at 1800 rpm for 1 minute.

Clean Up Ligation

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

Consumables

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

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. Vortex before use. 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. 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 LP MIDI plate.
3. Apply Microseal 'B' to the LP MIDI plate and shake the plate at 1800 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.

Wash

1. Place the LP 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 any 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' to the LP MIDI plate and shake the plate at 1800 rpm for 1 minute.
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 LP 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 cfDNA fragments flanked by index sequences and adapters required for cluster generation.

Consumables

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

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. 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, refer to 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 sample library.
3. In the post-amp area, save the following I-PCR program on the thermal cycler:
   - 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 of the assigned indexing primer (UPxx) to each well of the LS PCR plate. Apply a new tube cap to the indexing primer tube with a cap provided in your kit.
2. Add 20 µl EPM to each well.
3. Apply Microseal 'B' to the LS PCR plate and shake the plate at 1800 rpm for 1 minute.

   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 the 523 genes targeted by TruSight Oncology 500 ctDNA hybridize to DNA libraries prepared in *Index PCR* on page 12. Enrichment of targeted regions requires two hybridization steps. In this step, oligos hybridize to the DNA libraries overnight (8–24 hours).

Consumables

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

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Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
OPD2	-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 PCR plate was stored at -25°C to -15°C, prepare it as follows.
   a. Thaw at room temperature.

b. Centrifuge at 280 × g for 1 minute.
c. Pipette to mix and centrifuge.

3. Label a new 96-well PCR plate HYB1 (Hybridization 1).
4. Save the following HYB1 program on the thermal cycler:
   - 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 5 µl OPD2 to each well.
5. Apply Microseal 'B' to the HYB1 PCR plate and shake the plate at 1800 rpm for 1 minute.
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. Two 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

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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 and vortex to resuspend. If precipitate or the bead pellet is present, make sure to reach room temperature, 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 1 minute.

6. Incubate in a Hybex incubator at 57°C for 25 minutes.
7. Place on a magnetic stand for 2 minutes.
8. 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. Apply Microseal 'B' to the CAP1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.
   d. If the bead pellet is still present, remove the Microseal and pipette to mix. Make sure that all beads are resuspended, and then apply a new Microseal 'B'.
   e. Incubate in a Hybex incubator at 57°C for 10 minutes.
   f. Place on a magnetic stand for 2 minutes.
   g. While on the magnetic stand, use a pipette to 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. Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	8 Libraries (µl)
EE2	95	171
HP3	5	9

   - Prepare for a minimum of 3 libraries.
   - Discard any remaining elution 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' to the CAP1 MIDI plate and shake the plate at 1800 rpm for 1 minute.
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 1800 rpm for 1 minute.

Set Up Second Hybridization

This step binds targeted regions of the enriched 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 1.5–4 hours.

Consumables

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

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Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
OPD2	-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 precipitates are present, warm the tube in your hands, and then pipette to mix until the crystals are dissolved.

2. Save the following HYB2 program on the thermal cycler:
   - 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 5 µl OPD2 to each well.
4. Apply Microseal 'B' and shake the ELU1 PCR plate at 1800 rpm for 1 minute.
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 seal

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. 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, refer to the SDS at support.illumina.com/sds.html.

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 at room temperature and vortex before use.
SMB	2°C to 8°C	Bring to room temperature for 30 minutes and vortex to resuspend. If precipitate or the bead pellet is present, make sure to reach room temperature, 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 plate at 1800 rpm for 1 minute.
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' to the CAP2 MIDI plate and shake the plate at 1800 rpm for 2 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 any remaining elution 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' to the CAP2 MIDI plate and shake the plate at 1800 rpm for 1 minute.
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 plate at 1800 rpm for 1 minute.
10. Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, store ELU2 PCR 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

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. 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, refer to 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 PCR plate was stored at -25°C to -15°C, prepare it as follows.
   a. Thaw at room temperature.
   b. Centrifuge at 280 x g for 1 minute.
   c. Pipette to mix and centrifuge.
3. Save the following EL-PCR program on the thermal cycler:
   - 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 ELU2 PCR plate at 1800 rpm for 1 minute.
4. Briefly centrifuge at 280 x g.
5. Place on the preprogrammed thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

This step uses SPB to purify the enriched library from unwanted reaction components.

Consumables

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

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. Vortex before use. 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. Vortex for 1 minute before use.

2. Label a new 96-well MIDI plate BIND1.
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 and set aside.
2. Vortex SPB for 1 minute to resuspend the beads.
3. Add 110 µl SPB to each well of the BIND1 MIDI plate.
4. Transfer 50 µl of each library from the ELU2 PCR plate to the corresponding well of the BIND1 MIDI plate.
5. Apply Microseal ‘B’ to the BIND1 MIDI plate and shake the plate at 1800 rpm for 1 minute.
6. Incubate at room temperature for 5 minutes.

Wash

1. Place the BIND1 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 any residual supernatant from each well.

Elute

1. Remove the BIND1 MIDI plate from the magnetic stand.
2. Add 32 µl RSB to each well.
3. Apply Microseal 'B' to the BIND1 MIDI plate and shake the plate at 1800 rpm for 1 minute.
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 BIND1 MIDI plate to the corresponding well of the PL PCR plate.

SAFE STOPPING POINT

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

Quantify Libraries (Optional) (AccuClear)

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 AccuClear 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 the blanks in triplicate.
4. Run libraries in single replicates.
5. Determine the average relative fluorescence unit (RFU) for DNA standard and the blank.
6. Calculate the Normalized Standard RFU using the following formula.
   - Average Standard RFU - Average Blank RFU = Normalized Standard RFU

7. Calculate the Normalized RFU for each library using the following formula.
   - 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* on page 25. 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* on page 25.

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

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. 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, refer to the SDS at support.illumina.com/sds.html.

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.
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 PCR plate was stored at -25°C to -15°C, prepare it as follows.
   a. Thaw at room temperature.
   b. Centrifuge at 280 × g for 1 minute.
   c. Pipette to mix.
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.
2. Invert LNB1 after mixing to confirm bead pellet resuspension.

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

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

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

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

4. Vortex to mix.
5. 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

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

6. 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' to the BBN MIDI plate and shake the plate at 1800 rpm for 10 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 beads as follows.
   a. Remove the BBN MIDI plate from the magnetic stand.
   b. Add 45 µl LNW1 to each well.
   c. Apply Microseal 'B' to the BBN MIDI plate and shake the plate at 1800 rpm for 2 minutes.
   d. Place on a magnetic stand for 2 minutes.
   e. Remove and discard any residual 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' to the BBN MIDI plate and shake the plate 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 PCR 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

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

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.

Kit Contents

Make sure that you have the correct reagent kit identified in this section before proceeding to the protocol.

Library prep kit	Catalog #
TruSight Oncology 500 ctDNA Kit (48 Sample Library Prep Kit Only)	20039252

Library Prep

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

Quantity	Reagent	Description
2	UMI1	UMI Adapters v1

Box 2 - 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

Box 3 - 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

Quantity	Reagent	Description	Storage Temperature
1	TEB	TE Buffer	2°C to 8°C

Box 4 - 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 5 - 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

Quantity	Reagent	Description	Storage Temperature
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 6 - 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 7 - Enrichment (Post-Amp), Store at -25°C to -15°C

The TruSight Oncology 500 ctDNA Kit contains two of these boxes.

Quantity	Reagent	Description
1	OPD2	Oncology DNA Probes Master Pool 2

Consumables and Equipment

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

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes, nuclease-free	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier

Consumable	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
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
Ethanol (200 proof for molecular biology)	Sigma-Aldrich, part # E7023
[Optional] 96-well microplate, black, flat, clear bottom	Corning, part # 3904
[Optional] AccuClear Ultra High Sensitivity dsDNA Quantitation Kit with 1 DNA Standard	Biotium, catalog # 31029
[Optional] HS Large Fragment Kit	Agilent, part # DNF-493-1000 or DNF-493-0500

Equipment (Pre-Amp)

Equipment	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
[Optional] • 5200 Fragment Analyzer System or • 5300 Fragment Analyzer 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] Spectrophotometer	General lab supplier

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

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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