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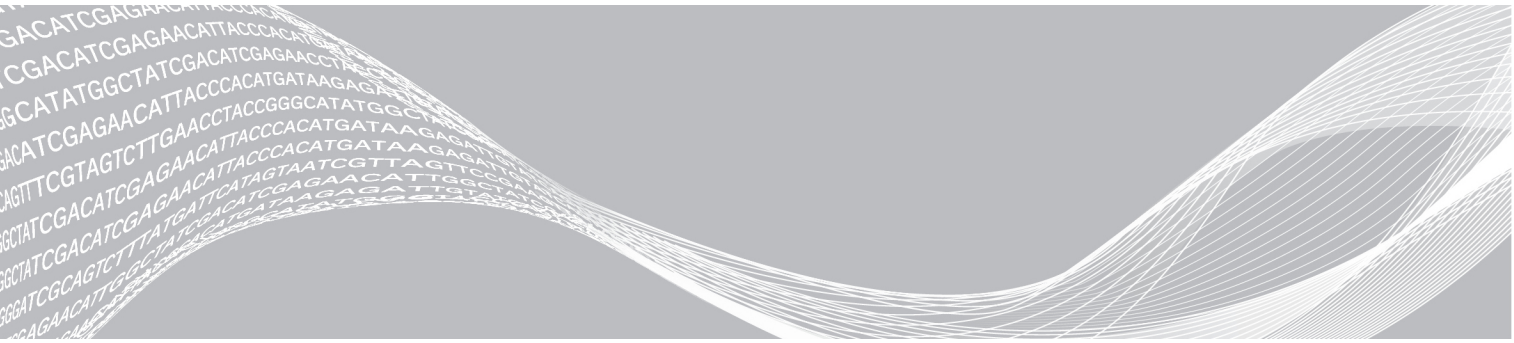
TruSight One Sequencing Panel Series

Reference Guide



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Revision History

Document	Date	Description of Change
Document # 15046431 v03	February 2018	• Removed reference to obsolete Protocol Guide from Additional Resources. • Removed acronyms unused in this document from the Acronyms table. • Updated procedure language to specify midi plates. • Updated TruSight One to TruSight One Series where appropriate.
Document # 15046431 v02	August 2017	• Updated protocol for TruSight One Expanded Sequencing Panel kits.
Document # 15046431 v01	January 2016	• Corrected storage of MiSeq Reagent Kit v3 Box 2 to 2°C to 8°C. • Updated design of workflow diagram. • Renamed and combined some procedures as needed to improve continuity. • Simplified consumables information at the beginning of each section. • Revised step-by-step instructions to be more succinct. • Removed reference to obsolete Experienced User Cards and added reference to new protocol guide and checklist. • Removed Preparing Your Libraries for Sequencing on a MiSeq section.
Part # 15046431 Rev. A	October 2013	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

This protocol explains how to prepare up to 36 indexed, paired-end libraries, followed by enrichment using the TruSight® One Sequencing Panel and reagents provided in Illumina TruSight One Sequencing Panel (Catalog # FC-141-1006, TG-141-1006, FC-141-1007, TG-141-1007) and TruSight One Expanded Sequencing Panel (Catalog # FC-141-1006-2007) kits. This protocol fragments and adds adapter sequences onto template DNA to generate indexed libraries that can be carried through enrichment for targeted resequencing applications.

The TruSight One Sequencing Panel protocol offers:

- Fast and easy sample preparation
  - Prepare up to 36 enriched libraries in approximately 1.5 days with approximately 5 hours of hands-on time
  - High throughput, automation-friendly procedures with no fragmentation bottlenecks
- Low DNA input and excellent data quality
  - Low input of 50 ng
  - Access precious samples with no effect on performance
  - Ability to archive samples for subsequent analysis
- High enrichment rates, low duplicates, and exceptional coverage uniformity
  - Efficient use of sequencing
  - Reliable variant calling
  - Reduced hands-on time with the most cost-effective, high-throughput workflow

DNA Input Recommendations

Using an enzymatic DNA fragmentation step allows TruSight One and TruSight One Expanded library preparations to be more sensitive to DNA input than mechanical fragmentation methods. Accurate quantification of the starting gDNA is essential to enrichment success.

Quantify the starting gDNA using a fluorometric-based method specific for double-stranded DNA (dsDNA) and run samples in triplicate. Avoid methods that measure total nucleic acid content, such as NanoDrop or other UV absorbance methods. Common contaminants such as ssDNA, RNA, and oligos are not substrates for the TruSight One Sequencing Panel.

The TruSight One protocol has been optimized for 50 ng of total gDNA. A higher mass input of gDNA can result in incomplete tagmentation and larger insert sizes, and can affect enrichment performance. Conversely, a lower mass input of gDNA or low-quality gDNA in the tagmentation reaction can generate smaller than expected insert sizes, which can be lost during subsequent cleanup steps and result in lower diversity.

To minimize gDNA sample input variability into the tagmentation step, perform a 2-step method of gDNA normalization. After the initial quantification, gDNA samples are normalized to 10 ng/µl. Samples are then quantified using a similar fluorometric-based method and normalized to a final 5 ng/µl.

Critical Steps for Successful Enrichment and Coverage

To ensure robust performance from the TruSight One Sequencing Panel, use a microheating system with a midi plate insert for the enrichment wash steps. The enrichment wash steps reduce nonspecific DNA binding and require that samples are maintained at the indicated temperature. Too low or too high temperatures can result in lower percent enrichments and decreased yields. If a microheating system is not available, a thermal cycler can be used with some modifications. See *Alternative Thermal Cycler Steps for Successful Enrichment* on page 25 for instructions using a thermal cycler.

Obtaining Desired Reads Per Sample

The number of resulting reads for each sample of a pool depend on the following factors:

- Accurate quantification of tagmented samples before pooling for enrichment. Inaccurate quantification can lead to uneven pooling between samples in the enrichment and can result in less than expected reads for a given sample.
- Accurate quantification of final enriched library pools. Use the same dilution of final library for both quantification and clustering. Inaccurate quantification can result in lower than targeted cluster densities, less reads passing filter and/or inefficient demultiplexing if overclustered.

Additional Resources

Visit the TruSight One Sequencing Panel support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

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

Resource	Description
Custom Protocol Selector	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 One Sequencing Panel Checklist (document # 1000000006696)	Provides a checklist of the protocol steps. The checklist is intended for experienced users. For new or less experienced users, see the TruSight One Sequencing Panel Reference Guide.

Chapter 2 Protocol

Introduction

This chapter describes the protocol for use with both TruSight One and TruSight One Expanded Panels.

- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Review Best Practices from the TruSight One Series Sequencing Panels support page on the Illumina website.
- Include a common index in each column. A common index facilitates pipetting operations when dispensing index adapters and pooling indexed libraries later in the protocol.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before beginning library prep. For more information, see the TruSight One Sequencing Panel support page.

Tips and Techniques

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 sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well 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.

- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

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

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

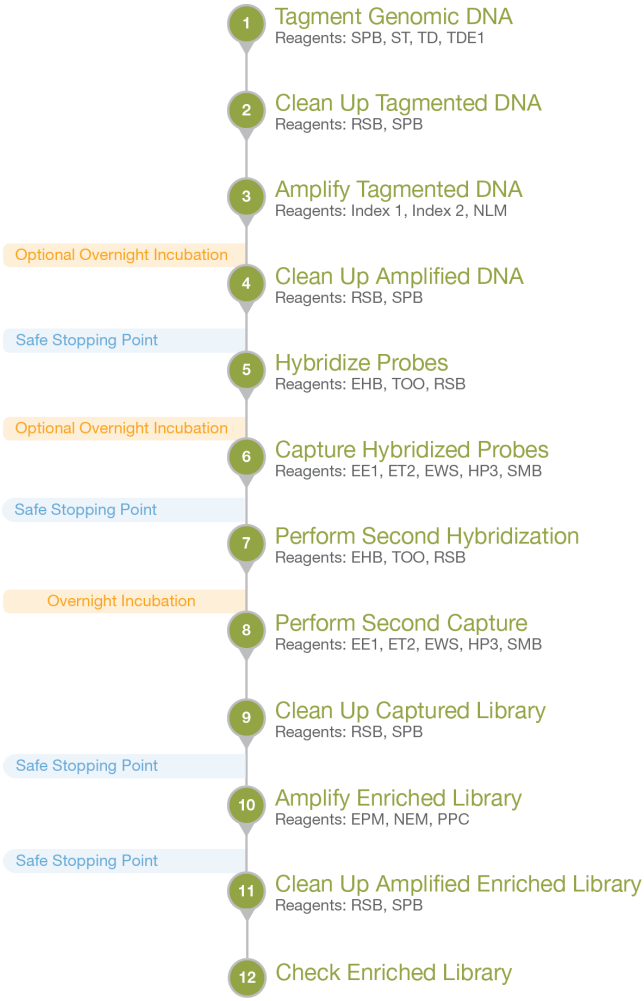
Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous.
- 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.

Library Prep Workflow

The following diagram illustrates the workflow using a TruSight One Sequencing Panel kit. Safe stopping points are marked between steps.

Figure 1 TruSight One Sequencing Panel Workflow



Tagment Genomic DNA

This step uses the Nextera transposome to tagment gDNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in a single step.

Consumables

- SPB (Sample Purification Beads)
- ST (Stop Tagment Buffer)
- TD (Tagment DNA Buffer)
- TDE1 (Tagment DNA Enzyme)
- gDNA (50 ng per sample)

- PCR-grade water
- Tris-HCl 10 mM, pH 8.5
- 96-well midi plate (1)
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
gDNA	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
TD	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
TDE1	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly. Set aside on ice.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Set aside at room temperature.
ST	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2 Preheat a microheating system with midi plate insert to 58°C.

Procedure

Quantify and Normalize gDNA

1 Quantify gDNA using a fluorometric method, such as QuantiFluor or Qubit.

2 Normalize gDNA in Tris-HCl 10 mM, pH 8.5 to 10 ng/µl.

3 Requantify the normalized gDNA using the same fluorometric quantification method.

4 Dilute the normalized gDNA in Tris-HCl 10 mM, pH 8.5 to a final volume of 10 µl at 5 ng/µl (50 ng total).

Tagment DNA

1 Add the following items in the order listed to each well of a new midi plate.
   - Normalized gDNA (10 µl)
   - TD (25 µl)
   - TDE1 (5 µl)
   - PCR-grade water (10 µl)

2 Seal the midi plate and shake at 1800 rpm for 1 minute.

3 Centrifuge at 280 × g for 1 minute.

4 Place the midi plate on the 58°C microheating system with the lid closed for 10 minutes.

5 Remove the seal and add 15 µl ST to each well.

6 Seal the midi plate and shake at 1800 rpm for 1 minute.

7 Centrifuge at 280 × g for 1 minute.

8 Incubate the midi plate at room temperature for 4 minutes.

Clean Up Tagmented DNA

This step uses SPB (Sample Purification Beads) to purify the tagmented DNA from the Nextera transposome. The cleanup step removes the Nextera transposome that can otherwise bind to DNA ends and interfere with downstream processes.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml 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 solution.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Remove seal and add 65 µl SPB to each well.

2 Seal the midi plate and shake at 1800 rpm for 1 minute.

3 Incubate the midi plate at room temperature for 8 minutes.

4 Centrifuge at 280 × g for 1 minute.

5 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Remove and discard all supernatant from each well.

7 Wash 2 times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

8 Use a 20 µl pipette to remove residual EtOH from each well.

9 Air-dry on the magnetic stand for 10 minutes.

10 Remove the midi plate from the magnetic stand.

11 Add 22.5 µl RSB to each well.

12 Seal the midi plate and shake at 1800 rpm for 1 minute.

13 Incubate the midi plate at room temperature for 2 minutes.

14 Centrifuge at 280 × g for 1 minute.

15 Place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

16 Remove seal and transfer 20 µl supernatant to the corresponding well of a new Hard-Shell PCR plate.

Amplify Tagmented DNA

This step amplifies purified tagmented DNA and adds index adapters using a 10-cycle PCR program. This PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequencing adapters required for cluster amplification.

Consumables

- Index 1 (i7) adapters and orange tube caps
- Index 2 (i5) adapters and white tube caps
- NLM (Library Amp Mix)
- 1.7 ml microcentrifuge tubes (1 per index adapter tube)
- Microseal 'A' film
- Microseal 'B' adhesive seal
- [Optional] TruSeq Index Plate Fixture Kit

NOTE

Use Microseal 'A' when sealing the plate before placing on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
Index adapters (i5 and i7)	-25°C to -15°C	Only remove adapters being used. Thaw at room temperature for 20 minutes. Vortex each tube to mix. Centrifuge briefly using a 1.7 ml Eppendorf tube.
NLM	-25°C to -15°C	Thaw on ice.

2 Save the following NLM AMP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 72°C for 3 minutes
- 98°C for 30 seconds
- 10 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

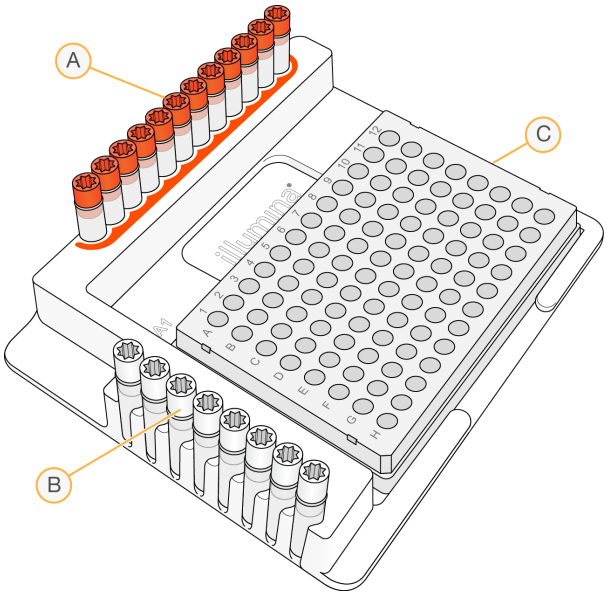
Procedure

1 Arrange Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture.

2 Arrange Index 2 (i5) adapters in rows A–H of the TruSeq Index Plate Fixture.

3 Place the plate on the TruSeq Index Plate Fixture.

Figure 2 TruSeq Index Plate Fixture (96 libraries)



A Columns 1–12: Index 1 (i7) adapters (orange caps)
B Rows A–H: Index 2 (i5) adapters (white caps)
C 96-well PCR plate

4 Using a multichannel pipette, add 5 µl of each Index 1 (i7) adapter down each column. Replace the cap on each i7 adapter tube with a new orange cap.

5 Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.

6 Add 20 µl NLM to each well.
The total volume per well is 50 µl.

7 Seal the PCR plate and shake at 1200 rpm for 1 minute.

8 Centrifuge at 280 × g for 1 minute.

9 Place the PCR plate on the preprogrammed thermal cycler and run the NLM AMP program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Amplified DNA

This step uses SPB (Sample Purification Beads) to purify the DNA library and remove unwanted products.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml 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
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Centrifuge at 280 × g for 1 minute.

2 Remove seal and transfer 50 µl supernatant to the corresponding well of a new midi plate (deep well plate).

3 Add 90 µl SPB to each well.

4 Seal the midi plate and shake at 1800 rpm for 1 minute.

5 Incubate the midi plate at room temperature for 10 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Remove seal and place on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Remove and discard all supernatant from each well.

9 Wash 2 times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

10 Use a 20 µl pipette to remove residual EtOH from each well.

11 Air-dry on the magnetic stand for 10 minutes.

12 Add 27.5 µl RSB to each well.

13 Seal the plate and shake at 1800 rpm for 1 minute.

14 Incubate the midi plate at room temperature for 2 minutes.

15 Centrifuge at 280 × g for 1 minute.

16 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

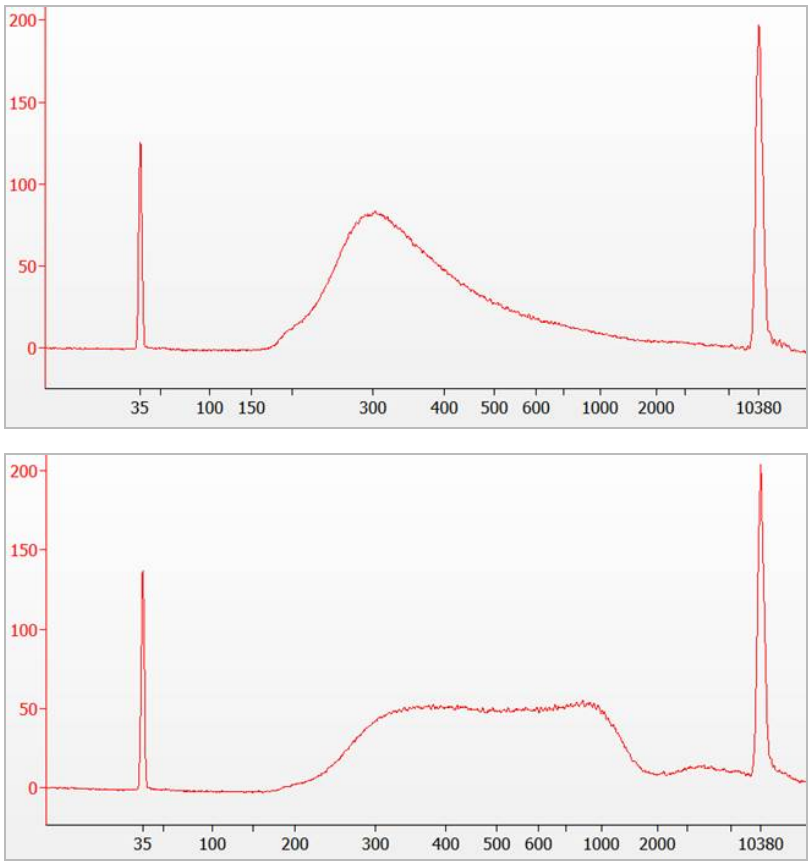
17 Transfer 25 µl supernatant to the corresponding well of a new Hard-Shell PCR plate.

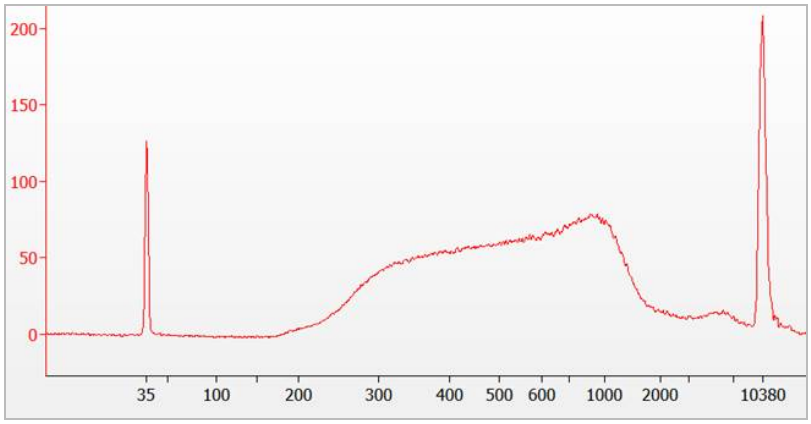
18 Quantify the library using a fluorometric method, such as QuantiFluor or Qubit.

19 [Optional] Run 1 µl of the library on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. Expect a distribution of DNA fragments with a size range from ~300 bp to ~1 kbp.

A sharp peak is not necessary, but most of the fragments must fall within the desired range. Traces can vary from library to library. The following traces show examples of possible distributions, but are not inclusive of successful libraries.

Figure 3 Examples of Post-PCR, Pre-Enriched Library Distribution





SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 14 days.

Hybridize Probes

This step combines DNA libraries containing unique indexes into a single pool, and then binds targeted regions of the DNA with capture probes.

Consumables

- EHB (Enrichment Hybridization Buffer)
- TOO (TruSight One Oligos) or TOE (TruSight One Expanded Oligos)
- RSB (Resuspension Buffer)
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seal
- [Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) (1 per pooled sample)

About Reagents

- Before using EHB, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
TOO or TOE	-25°C to -15°C	Thaw at room temperature.
EHB	-25°C to -15°C	Thaw at room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Save the NRC HYB program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 95°C for 10 minutes
- 94°C for 1 minute
- 92°C for 1 minute

- 90°C for 1 minute
- 88°C for 1 minute
- 86°C for 1 minute
- 84°C for 1 minute
- 82°C for 1 minute
- 80°C for 1 minute
- 78°C for 1 minute
- 76°C for 1 minute
- 74°C for 1 minute
- 72°C for 1 minute
- 70°C for 1 minute
- 68°C for 1 minute
- 66°C for 1 minute
- 64°C for 1 minute
- 62°C for 1 minute
- 60°C for 1 minute
- Hold at 58°C

Pool Libraries

1 Combine 500 ng of each DNA library. Make sure that each library has a unique dual index combination.

Library Pool Complexity	Total DNA Library Mass (ng)	Library Pool Complexity	Total DNA Library Mass (ng)
1-plex	500	7-plex	3500
2-plex	1000	8-plex	4000
3-plex	1500	9-plex	4500
4-plex	2000	10-plex	5000
5-plex	2500	11-plex	5500
6-plex	3000	12-plex	6000

- If the total volume is > 40 µl, use a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) to concentrate the pooled sample to 40 µl.
  - If you are using a vacuum concentrator, use a no heat setting and a medium drying rate.
  - If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), rinsing the device before use is not required. Most volume filters through in 5 minutes. Up to 30 minutes might be needed, depending on the starting volume.
- If the total volume is < 40 µl, increase the volume to 40 µl with RSB.

Procedure

1 Add the following items in the order listed to each well of a new Hard-Shell PCR plate.
- DNA library sample or pool (40 µl)
- EHB (50 µl)
- TOO or TOE (10 µl)

2 Seal the plate and shake at 1200 rpm for 1 minute.

3 Centrifuge at 280 × g for 1 minute.

4 Place PCR plate on the preprogrammed thermal cycler and run the NRC HYB program. Each well contains 100 µl.

5 Keep at the 58°C holding temperature for at least 90 minutes and up to 24 hours.

NOTE

Hybridizing overnight can have a positive correlation with increasing enrichment percentages.

Capture Hybridized Probes

This process mixes the DNA library with capture probes to targeted regions of interest. Next, Streptavidin Magnetic Beads (SMB) are used to capture hybridized probes. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well Hard-Shell 0.3 ml PCR plate
- 96-well midi plate
- 1.7 ml microcentrifuge tube
- Microseal 'B' adhesive seals

About Reagents

- EWS can be cloudy after reaching room temperature.
- Vortex EWS before use.
- Make sure that you use SMB (2 ml tube) and not SPB (15 ml tube) for this procedure.
- Invert and vortex SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.

2 Preheat a microheating system with midi plate insert to 50°C.

Procedure

First Bind

1 Centrifuge at 280 × g for 1 minute.

2 Remove seal and transfer all volumes to the corresponding well of a new midi plate.

3 Add 250 µl SMB to each well.

4 Seal the midi plate and shake at 1200 rpm for 5 minutes.

5 Incubate the midi plate at room temperature for 25 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Remove and discard all supernatant from each well.

9 Remove the midi plate from the magnetic stand.

First Wash

1 Wash two times as follows.

   a Add 200 µl EWS to each well.
   b Seal the midi plate and shake at 1800 rpm for 4 minutes.
   c Remove seal and pipette to completely resuspend the bead pellet further.
   d Seal and place the midi plate on the 50°C microheating system with the lid closed for 30 minutes.
   e Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).
   f Remove and discard all supernatant from each well.
   g Remove the midi plate from the magnetic stand.

First Elution

1 Create elution premix by combining the following volumes per sample in a 1.7 ml microcentrifuge tube,

and then vortex.
- EE1 (28.5 µl)
- HP3 (1.5 µl)

2 Add 23.5 µl elution premix to each well.

3 Seal the midi plate and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

7 Transfer 21 µl supernatant to the corresponding well of a new Hard-Shell PCR plate.

8 Add 4 µl ET2 to each well.

9 Seal the plate and shake at 1200 rpm for 1 minute.

10 Centrifuge at 280 × g for 1 minute.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to seven days.

Perform Second Hybridization

This step binds targeted regions of the enriched DNA with capture probes a second time. This second hybridization ensures high specificity of the captured regions.

Consumables

- EHB (Enrichment Hybridization Buffer)
- TOO (TruSight One Oligos) or TOE (TruSight One Expanded Oligos)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive seals

About Reagents

- Before using EHB, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
TOO or TOE	-25°C to -15°C	Thaw at room temperature.
EHB	-25°C to -15°C	Thaw at room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

Procedure

1 Remove seal and add the following reagents in the order listed to each sample well.
- RSB (15 µl)
- EHB (50 µl)
- TOO or TOE (10 µl)

2 Seal the midi plate and shake at 1200 rpm for 1 minute.

3 Centrifuge at 280 × g for 1 minute.

4 Place the midi plate on the preprogrammed thermal cycler and run the NRC HYB program. Each well contains 100 µl.

5 Keep at the 58°C holding temperature for at least 14.5 hours and up to 24 hours.

Perform Second Capture

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

Consumables

- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well midi plates (2)
- 1.7 ml microcentrifuge tube
- Microseal 'B' adhesive seals

About Reagents

- EWS can be cloudy after reaching room temperature.
- Vortex EWS before use.
- Invert SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

Item	Storage	Instructions
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.

2 Preheat a microheating system with midi plate insert to 50°C.

Procedure

Second Bind

1 Centrifuge at 280 × g for 1 minute.

2 Remove seal and transfer supernatant to the corresponding well of a new midi plate.

3 Add 250 µl SMB to each well.

4 Seal the plate and shake at 1200 rpm for 5 minutes.

5 Incubate the midi plate at room temperature for 25 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Remove and discard all supernatant from each well.

9 Remove the midi plate from the magnetic stand.

Second Wash

1 Wash two times as follows.

   a Add 200 µl EWS to each well.
   b Seal the midi plate and shake at 1800 rpm for 4 minutes.
   c Remove the seal and pipette to completely resuspend the bead pellet further.
   d Seal and place the midi plate on the 50°C microheating system with the lid closed for 30 minutes.
   e Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).
   f Remove and discard all supernatant from each well.
   g Remove the midi plate from the magnetic stand.

Second Elution

1 Create elution premix by combining the following volumes per sample in a 1.7 ml microcentrifuge tube, and then vortex:
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)

2 Add 23.5 µl elution premix to each well.

3 Seal the midi plate and shake at 1800 rpm for 2 minutes.

4 Incubate the midi plate at room temperature for 2 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Remove the seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

7 Transfer 21 µl supernatant to the corresponding well of a new midi plate.

8 Add 4 µl ET2 to each well.
The total volume per well in the new midi plate is 25 µl.

9 Seal the midi plate and shake at 1800 rpm for 1 minute.

10 Centrifuge at 280 × g for 1 minute.

Clean Up Captured Library

This step uses SPB (Sample Purification Beads) to purify the captured library before PCR amplification.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml 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 solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Remove seal and add 45 µl SPB to each well.
The total volume per well is 70 µl.

2 Seal the midi plate and shake at 1800 rpm for 1 minute.

3 Incubate the midi plate at room temperature for 10 minutes.

4 Centrifuge at 280 × g for 1 minute.

5 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Remove and discard all supernatant from each well.

7 Wash two times as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

8 Use a 20 µl pipette to remove residual EtOH from each well.

9 Air-dry on the magnetic stand for 10 minutes.

10 Add 27.5 µl RSB to each well.

11 Seal the midi plate and shake at 1800 rpm for 1 minute.

12 Incubate at room temperature for 2 minutes.

13 Centrifuge at 280 × g for 1 minute.

14 Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

15 Transfer 25 µl supernatant to the corresponding well of a new Hard-Shell PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to seven days.

Amplify Enriched Library

This step uses a 10-cycle PCR program to amplify the enriched library.

Consumables

- NEM (Enrichment Amp Mix)
- PPC (PCR Primer Cocktail)
- Microseal 'A' film
- Microseal 'B' adhesive seal

NOTE

Use Microseal 'A' when sealing the plate before placing on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
NEM	-25°C to -15°C	Thaw on ice.
PPC	-25°C to -15°C	Thaw on ice.

2 Save the following NEM AMP10 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 98°C for 30 seconds
   - 10 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 Remove seal and add 5 µl PPC to each well.

2 Add 20 µl NEM to each well.
The total volume per well is 50 µl.

3 Apply the seal and shake at 1200 rpm for 1 minute.

4 Centrifuge at 280 × g for 1 minute.

5 Place the midi plate on the preprogrammed thermal cycler and run the NEM AMP10 program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days.

Clean Up Amplified Enriched Library

This step uses SPB (Sample Purification Beads) to purify the enriched library and remove unwanted products.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml 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
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Centrifuge at 280 × g for 1 minute.

2 Remove seal and transfer 50 µl to the corresponding well of a new midi plate.

3 Add 90 µl SPB to each well.

4 Seal the midi plate and shake at 1800 rpm for 1 minute.

5 Incubate the midi plate at room temperature for 10 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Remove the seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Remove and discard all supernatant from each well.

9 Wash 2 times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

10 Use a 20 µl pipette to remove residual EtOH from each well.

11 Air-dry on the magnetic stand for 10 minutes.

12 Add 32.5 µl RSB to each well.

13 Seal the midi plate and shake at 1800 rpm for 1 minute.

14 Incubate the midi plate at room temperature for 2 minutes.

15 Centrifuge at 280 × g for 1 minute.

16 Place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

17 Transfer 30 µl supernatant to the corresponding well of a new Hard-Shell PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to seven days.

Check Enriched Libraries

Perform the following procedures to check the quality of the enriched library.

Quantify Libraries

Accurately quantify DNA libraries to ensure optimum cluster densities on the flow cell.

Use a fluorometric dsDNA assay to quantify dsDNA libraries. Other techniques can introduce contamination such as RNA and proteins. Use a spectrofluorometer for DNA-specific quantification. Spectrophotometry can also measure RNA and yield values that are too high.

1 Dilute the postenriched library before quantification as follows:
   - For 3-plex to 12-plex enrichments, dilute by adding 2 µl library to 28 µl RSB in a new tube or well. Use this dilution for quantification and quality assessment, as well as sequencing.
   - For 1-plex or 2-plex enrichments, a dilution is not needed.

2 Quantify using a fluorometric method.
Use the following formula to convert from ng/µl to nM. Assume a 650 bp library size or calculate based on the average size of the enriched library.

$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol * average library size)}}$	x 10^6	= concentration in nM

For example:

$\frac{\text{(15 ng/µl)}}{\text{(660 g/mol * 650)}}$	x 10^6	= 34.9 nM

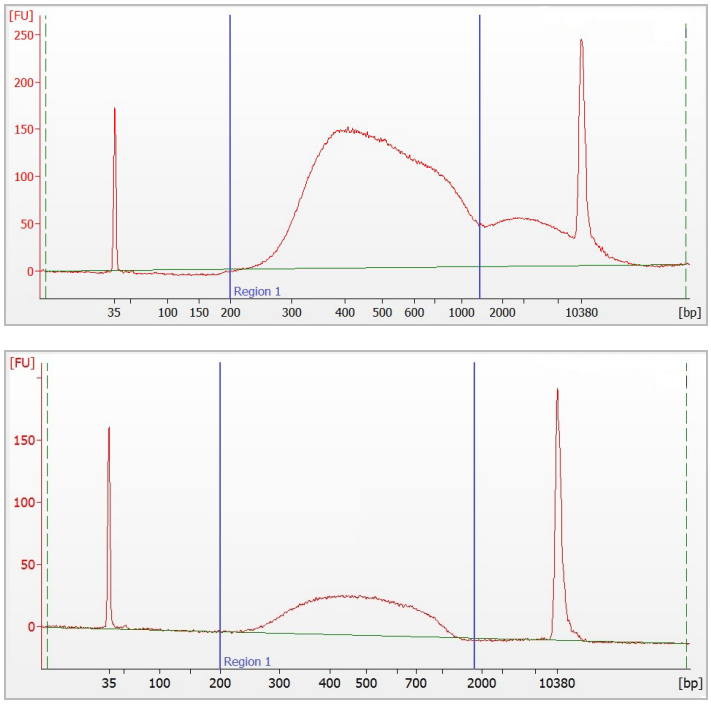
3 Do the following:
- To assess library quality, proceed to the next step in the workflow.
- To skip library quality assessment, proceed to cluster generation. For more information, see the system guide for your Illumina sequencing instrument.

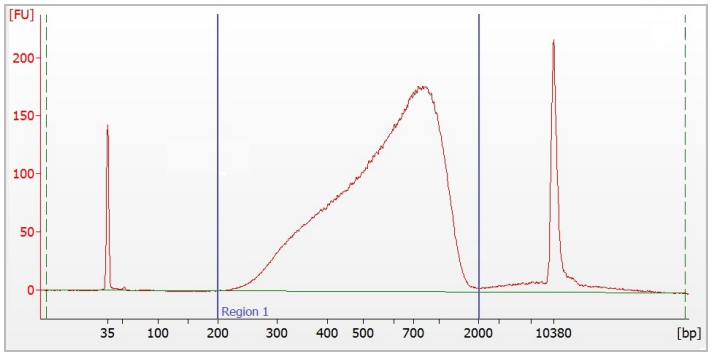
Alternatively, you can quantify libraries using qPCR according to the *Sequencing Library qPCR Quantification Guide (part # 11322363)*.

Assess Quality **[Optional]**

1 Run 1 µl post enriched library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.
Expect a distribution of DNA fragments with a size range from ~200 bp to ~1 kbp. Depending on the level of indexing, insert size distribution can vary slightly. However, the sample peak must not be significantly shifted compared to the following example.

Figure 4 Example Post Enrichment Library Distributions





NOTE

The blue lines indicate the boundaries that were manually created to determine average library size. In the first example, a second minor peak at ~2000 bp is visible. Do not include minor peaks in the determination of average library size. The presence of these larger fragments does not affect downstream clustering and sequencing of your enriched library.

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
EE1	Enrichment Elution Buffer 1
EHB	Enrichment Hybridization Buffer
ET2	Elute Target Buffer 2
EWS	Enrichment Wash Solution
HP3	2N NaOH
NEM	Enrichment Amp Mix
NLM	Library Amp Mix
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
ST	Stop Tagment Buffer
TD	Tagment DNA Buffer
TDE1	Tagment DNA Enzyme TDE
TOO	TruSight One Oligos
TOE	TruSight One Expanded Oligos

Alternative Thermal Cycler Steps for Successful Enrichment

If using a thermal cycler instead of a microheating system during enrichment wash steps, perform the following steps.

1 Save the following WASH program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 42°C for 30 minutes

2 Transfer samples and beads resuspended in EWS to a PCR plate (~200 µl).

3 Place on a thermal cycler and run the WASH program.

4 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

5 Remove and discard all supernatant from each well.

6 Remove from the magnetic stand.

7 Add 200 µl EWS to each well.

8 Shake at 1800 rpm for 4 minutes.

9 Pipette to resuspend the bead pellet further.

10 Repeat steps 3–6 for a total of two washes.

11 Continue to the elution step.

Kit Contents

Make sure that you have all reagents identified in this section before proceeding to the library preparation procedures. TruSight One kits are available in the following configurations.

Kit Name	Catalog #	*TG Catalog #	Number of Samples
TruSight One Sequencing Panel (9 Samples)	FC-141-1006	TG-141-1006	9
TruSight One Sequencing Panel (36 Samples)	FC-141-1007	TG-141-1007	36
TruSight One Expanded Sequencing Panel (36 Samples)	FC-141-2007	N/A	36

* Consumables labeled TG include features to help reduce the frequency of revalidation. These consumables are available with a supply agreement and require providing a binding forecast. For more information, contact your Illumina representative.

Note regarding biomarker patents and other patents unique to specific uses of products.

Some genomic variants, including some nucleic acid sequences, and their use in specific applications might be protected by patents. Customers are advised to determine whether they are required to obtain licenses from the party that owns or controls such patents to use the product for their specific application.

TruSight One Sequencing Panel Contents (9 Samples) (FC-141-1006, TG-141-1006)

The TruSight One Sequencing Panel includes MiSeq Reagent v3 kits.

Box 1 - Rapid Capture Reagents

Quantity	Reagent	Description	Storage Temperature
1	SPB	Sample Purification Beads	2°C to 8°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
1	ET2	Elute Target Buffer 2	2°C to 8°C
1	ST	Stop Tagment Buffer	15°C to 30°C

Box 2 - Rapid Capture Reagents, Store at -25°C to -15°C

Quantity	Reagent	Description
1	TDE1	Tagment DNA Enzyme
1	EE1	Enrichment Elution Buffer 1
1	TD	Tagment DNA Buffer
1	RSB	Resuspension Buffer
1	NLM	Nextera Library Amplification Mix
1	EHB	Enrichment Hybridization Buffer
1	EWS	Enrichment Wash Solution
1	HP3	2N NaOH
1	PPC	PCR Primer Cocktail
2	NEM	Nextera Enrichment Amplification Mix

Box 3 - Indices, Store at -25°C to -15°C

Quantity	Reagent
2	i5 Index Primers, E503, E504
3	i7 Index Primers, N701, N705, N709

Box 4 - Oligos, Store at -25°C to -15°C

Quantity	Reagent
1	TruSight One Content Set

MiSeq Reagent Kit v3, Box 1, Store at -25°C to -15°C

Quantity	Component
3	HT1 (Hybridization Buffer)
3	Reagent Cartridge

MiSeq Reagent Kit v3, Box 2, Store at 2°C to 8°C

Quantity	Component
3	MiSeq Flow Cell
3	Reagent Cartridge

TruSight One Sequencing Panel or TruSight One Expanded Sequencing Panel Contents (36 Samples) (FC-141-1007, TG-141-1007) or (FC-141-2007)

Box 1 - Rapid Capture Reagents

Quantity	Reagent	Description	Storage Temperature
2	SPB	Sample Purification Beads	2°C to 8°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
1	ET2	Elute Target Buffer 2	2°C to 8°C
1	ST	Stop Tagment Buffer	15°C to 30°C

Box 2 - Rapid Capture Reagents, Store at -25°C to -15°C

Quantity	Reagent	Description
2	TDE1	Tagment DNA Enzyme
1	EE1	Enrichment Elution Buffer 1
1	TD	Tagment DNA Buffer
1	RSB	Resuspension Buffer
2	NLM	Nextera Library Amplification Mix
1	EHB	Enrichment Hybridization Buffer
1	EWS	Enrichment Wash Solution
1	HP3	2N NaOH
1	PPC	PCR Primer Cocktail
2	NEM	Nextera Enrichment Amplification Mix

Box 3 - Indices, Store at -25°C to -15°C

Quantity	Reagent
4	i5 Index Primers, E502 to E505
12	i7 Index Primers, N701 to N712

Box 4 - Oligos, Store at -25°C to -15°C

Quantity	Reagent
1	TruSight One Content Set or TruSight One Expanded Content Set

Consumables and Equipment

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

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

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
Adhesive seal roller	General lab supplier
96-well storage plates, round well, 0.8 ml (midi plate)	Fisher Scientific, part # AB-0859
Hard-Shell 96-well PCR Plates	Bio-Rad, part # HSP-9601
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Microseal 'A' film	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
PCR-grade water	General lab supplier
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)*	Millipore, part # UFC503008
[Optional] DNA 1000 Kit	Agilent Technologies, part # 5067-1504
[Optional] High Sensitivity DNA Kit	Agilent Technologies, part # 5067-4626

* Use to concentrate a pooled library. Otherwise, use a vacuum concentrator.

Equipment

Equipment	Supplier
DNA Engine Multi-Bay Thermal Cycler See *Thermal Cyclers* on page 30.	Bio-Rad, part # PTC-0240G or PTC-0220G, with Alpha Unit, part # ALS-1296GC
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
Magnetic stand-96	Life Technologies, part # AM10027
Microcentrifuge	General lab supplier

Equipment	Supplier
Microheating System-SciGene TruTemp Heating System	Illumina, catalog # • SC-60-503 (115 V) or • SC-60-504 (220 V)
Microplate centrifuge	General lab supplier
Midi plate insert for microheating system	Illumina, catalog # BD-60-601
Fluorometric quantification with dsDNA binding dye reagents	General lab supplier
Vortexer	General lab supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA
[Optional] TruSeq Index Plate Fixture Kit[1]	Illumina, catalog # FC-130-1005
[Optional] Vacuum concentrator[2]	General lab supplier

[1] Reusable and recommended for setting up indexed adapters.

[2] Use to concentrate a pooled library. Alternatively, use Amicon Ultra-0.5 centrifugal filter units.

Thermal Cyclers

The following table lists the recommended settings for the recommended thermal cycler, and other comparable models. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Index Sequences

The Illumina dual-index strategy adds two 8-base indexes, Index 1 (i7) and Index 2 (i5), to each sample.

- N refers to Nextera
- E refers to Enrichment
- 7 refers to Index 1 (i7)
- 5 refers to Index 2 (i5)
- 01–12 refers to the Index number

Use the following bases for entry on your sample sheet.

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	E502*	CTCTCTAT
N702*	CGTACTAG	E503	TATCCTCT
N703*	AGGCAGAA	E504	AGAGTAGA
N704*	TCCTGAGC	E505*	GTAAGGAG
N705	GGACTCCT		
N706*	TAGGCATG		
N707*	CTCTCTAC		
N708*	CAGAGAGG		
N709	GCTACGCT		
N710*	CGAGGCTG		
N711*	AAGAGGCA		
N712*	GTAGAGGA		

* Only available in the TruSight One Sequencing Panel Kit (36 Samples).

NOTE

The E500 series Index 2 (i5) sequences in the TruSight One series kits are identical to S500 series Index 2 (i5) sequences in other kits. However, the Index 2 (i5) adapters are not interchangeable across kits.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

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**.

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