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# Table of Contents

Table of Contents .....	iii
Revision History .....	iv
<b>Chapter 1 Overview .....</b>	<b>1</b>
Introduction .....	2
DNA Input Recommendations .....	3
Additional Resources .....	4
<b>Chapter 2 Protocol .....</b>	<b>5</b>
Introduction .....	6
Tips and Techniques .....	7
Library Prep Workflow .....	8
Tagment Genomic DNA .....	9
Clean Up Tagmented DNA .....	11
Amplify Tagmented DNA .....	13
Clean Up Amplified DNA .....	15
Hybridize Probes .....	17
Capture Hybridized Probes .....	19
Perform Second Hybridization .....	21
Perform Second Capture .....	22
Clean Up Captured Library .....	24
Amplify Enriched Library .....	26
Clean Up Amplified Enriched Library .....	27
Check Enriched Libraries .....	29
<b>Appendix A Supporting Information .....</b>	<b>31</b>
Introduction .....	32
Acronyms .....	33
Kit Contents .....	34
Consumables and Equipment .....	37
Index Sequences .....	40
<b>Technical Assistance .....</b>	<b>41</b>

# Revision History

Document	Date	Description of Change
Document # 15063774 v01	January 2016	<ul style="list-style-type: none"><li>• Updated the kit name from TruSight Cardio Sequencing Panel to TruSight Cardio Sequencing kit.</li><li>• Removed reference to sample sheet and data analysis resources and added reference to Custom Protocol Selector.</li><li>• Added information on pooling preparation.</li></ul>
Document # 15063774 Rev. B	May 2015	<ul style="list-style-type: none"><li>• Added kit content information for sequencing on the NextSeq.</li><li>• Removed the <i>Prepare Libraries for Sequencing</i> section. Content from this section can be found in the system guide for your Illumina sequencing platform.</li><li>• Updated the sequencing guidelines.</li><li>• The <i>Hybridize Additional Probes</i> section is now called <i>Perform Second Hybridization</i>.</li><li>• The <i>Capture Additional Hybridized Probes</i> section is now called <i>Perform Second Capture</i>.</li></ul>
Document # 15063774 Rev. A	March 2015	Initial release.

# Overview

Introduction .....	2
DNA Input Recommendations .....	3
Additional Resources .....	4



## Introduction

This protocol explains how to prepare up to 48 indexed paired-end libraries followed by enrichment using the TruSight Cardio Sequencing Kit.

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 Cardio protocol offers:

- ▶ Fast and easy sample preparation
  - ▶ Prepare up to 12 or 48 enriched libraries in approximately 1.5 days, with approximately 5 hours of hands-on time
- ▶ Low DNA input and excellent data quality
  - ▶ Excellent data quality with low input of 50 ng
  - ▶ Access precious samples with no affect 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 Cardio library preparation 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 Cardio Sequencing Kit.

- ▶ Make sure that the starting DNA does not contain more than 1 mM EDTA and is free of organic contaminants, such as phenol and ethanol.
- ▶ DNA samples can contain substances that interfere with the Nextera tagmentation reaction and result in unexpected library insert sizes. An additional sample cleanup and quantification step before library preparation ensures optimal conditions for library preparation.

The TruSight Cardio 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.

## Additional Resources

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

Resource	Description
Custom Protocol Selector	<a href="http://support.illumina.com/custom-protocol-selector.html">http://support.illumina.com/custom-protocol-selector.html</a> 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.
<i>TruSight Cardio Sequencing Kit Protocol Guide (document # 15069812)</i>	Provides only protocol instructions. The protocol guide is intended for experienced users. For lesser experienced users, see the TruSight Cardio Sequencing Kit Reference Guide.
<i>TruSight Cardio Sequencing Kit Checklist (document # 15069811)</i>	Provides a checklist of the protocol steps. The checklist is intended for experienced users. For new or less experienced users, see the TruSight Cardio Sequencing Kit Reference Guide.
<i>Nextera Rapid Capture Enrichment Low-Plex Pooling Guidelines Technical Note</i>	Provides pooling guidelines and dual indexing strategies for Nextera Rapid Capture Enrichment library preparation.



# Protocol

Introduction .....	6
Tips and Techniques .....	7
Library Prep Workflow .....	8
Tagment Genomic DNA .....	9
Clean Up Tagmented DNA .....	11
Amplify Tagmented DNA .....	13
Clean Up Amplified DNA .....	15
Hybridize Probes .....	17
Capture Hybridized Probes .....	19
Perform Second Hybridization .....	21
Perform Second Capture .....	22
Clean Up Captured Library .....	24
Amplify Enriched Library .....	26
Clean Up Amplified Enriched Library .....	27
Check Enriched Libraries .....	29



## Introduction

This chapter describes the TruSight Cardio protocol.

- ▶ Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- ▶ Review Best Practices from the TruSight Cardio support page on the Illumina website.

## Prepare for Pooling

If you plan to pool libraries, record information about your samples before beginning library prep. Different methods are available depending on the sequencing instrument you are using. See the TruSight Cardio Sequencing Kit support page for more information.

## 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 effective for thermal cycling and easy to cut when using fewer than 96 wells.

### 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.

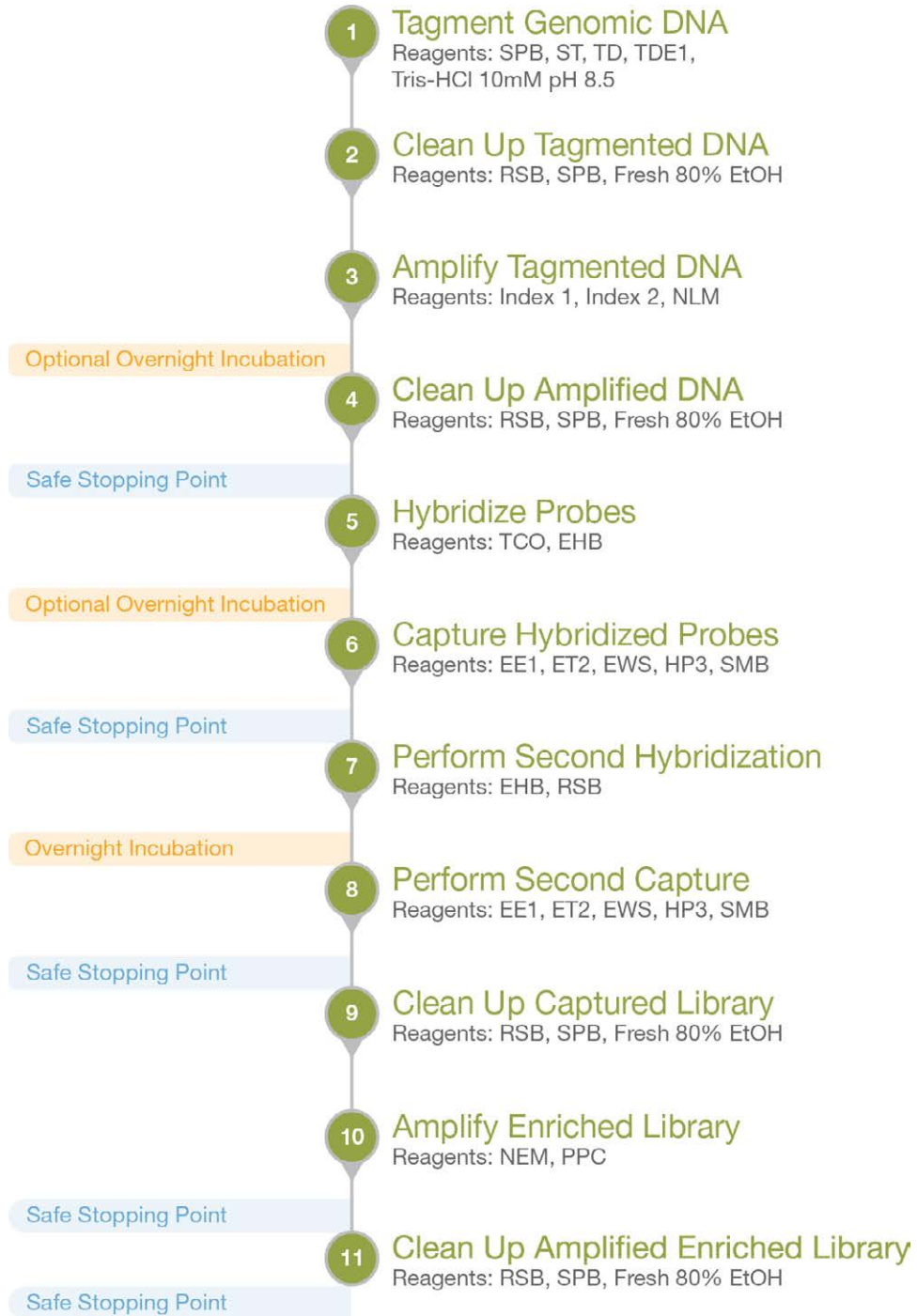
### Handling Beads

- ▶ Pipette bead suspension slowly.
- ▶ When mixing, mix thoroughly.
- ▶ If beads are aspirated into the 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 appropriate magnet for the plate.
  - ▶ Dispense liquid so that beads on the side of the wells are wetted.
  - ▶ Keep the plate on the magnet until the instructions specify to remove it.
  - ▶ Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

## Library Prep Workflow

The following diagram illustrates the workflow using a TruSight Cardio Sequencing Kit. Safe stopping points are marked between steps.

Figure 1 TruSight Cardio Sequencing Kit 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)
- ▶ 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 (15 μl)
- 2 Shake at 1800 rpm for 1 minute.
- 3 Centrifuge at 280 × g for 1 minute.

- 4 Place on the 58°C microheating system with the lid closed for 10 minutes.
- 5 Add 15 µl ST to each well.
- 6 Shake at 1800 rpm for 1 minute.
- 7 Centrifuge at 280 × g for 1 minute.
- 8 Incubate 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 Add 65  $\mu$ l SPB to each well.
- 2 Shake at 1800 rpm for 1 minute.
- 3 Incubate at room temperature for 8 minutes.
- 4 Centrifuge at  $280 \times g$  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 2 times as follows.
  - a Add 200  $\mu$ l 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  $\mu$ l pipette to remove residual EtOH from each well.
- 9 Air-dry on the magnetic stand for 10 minutes.
- 10 Remove from the magnetic stand.
- 11 Add 22.5  $\mu$ l RSB to each well.
- 12 Shake at 1800 rpm for 1 minute.

- 13 Incubate at room temperature for 2 minutes.
- 14 Centrifuge at  $280 \times g$  for 1 minute.
- 15 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 16 Transfer 20  $\mu\text{l}$  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.

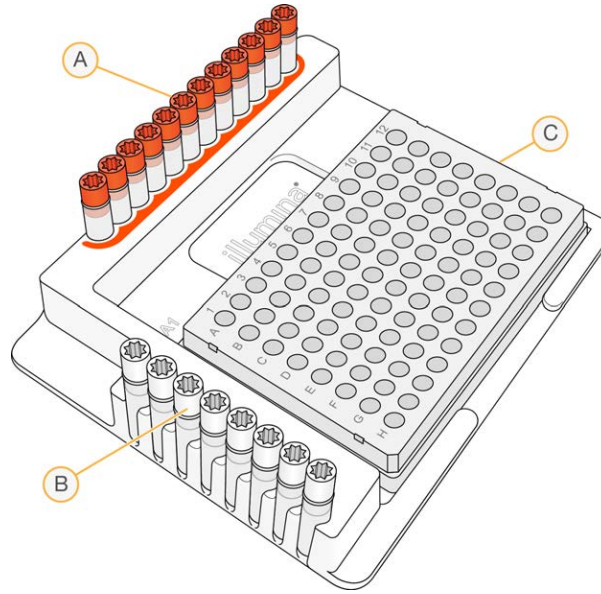
- 2 Save the following NLM AMP program on the thermal cycler:

- ▶ 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 plate

- 4 Using a multichannel pipette, add 5  $\mu$ l 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  $\mu$ l 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  $\mu$ l NLM to each well.
- 7 Shake at 1200 rpm for 1 minute.
- 8 Centrifuge at 280  $\times$  g for 1 minute.
- 9 Place 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

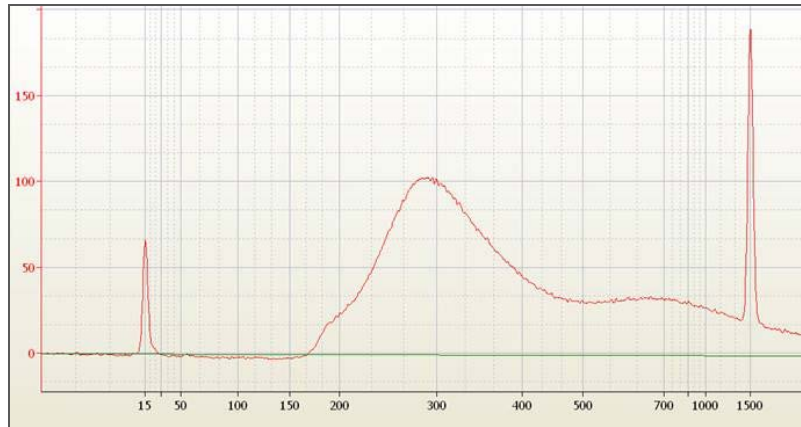
- 1 Centrifuge at  $280 \times g$  for 1 minute.
- 2 Transfer 50  $\mu$ l supernatant to the corresponding well of a new midi plate.
- 3 Add 90  $\mu$ l SPB to each well.
- 4 Shake at 1800 rpm for 1 minute.
- 5 Incubate at room temperature for 10 minutes.
- 6 Centrifuge at  $280 \times g$  for 1 minute.
- 7 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  $\mu$ l 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  $\mu$ l pipette to remove residual EtOH from each well.
- 11 Air-dry on the magnetic stand for 10 minutes.
- 12 Add 27  $\mu$ l RSB to each well.

- 13 Shake at 1800 rpm for 1 minute.
- 14 Incubate at room temperature for 2 minutes.
- 15 Centrifuge at  $280 \times g$  for 1 minute.
- 16 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 17 Transfer 25  $\mu$ l 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  $\mu$ l 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** Example of Post-PCR, Pre-Enriched Library Distribution



#### NOTE

The sample peak must not be significantly shifted compared to the example shown in Figure 3, but traces can differ depending on sample quality. A larger peak distribution (> 350 bp) can indicate > 50 ng gDNA input going into tagmentation, which can cause lower on-target reads. A smaller sample peak distribution (< 225 bp) can indicate < 50 ng gDNA or low quality gDNA, which can cause reduced library diversity or elevated duplicates.

### SAFE STOPPING POINT

If you are stopping, seal the plate and store at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  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)
- ▶ TCO (TruSight Cardio 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
TCO	-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
  - ▶ 18 cycles of 1 minute each, starting at 94°C, then decreasing 2°C per cycle
  - ▶ Hold at 58°C

## Pool Libraries

- 1 Combine 500 ng of each DNA library. Make sure that each library has a unique index. Up to 12 DNA libraries can be pooled before adding capture probes.

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  $\mu$ l, increase the volume to 40  $\mu$ l 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  $\mu$ l)
  - ▶ EHB (50  $\mu$ l)
  - ▶ TCO (10  $\mu$ l)
- 2 Shake at 1200 rpm for 1 minute.
- 3 Centrifuge at  $280 \times g$  for 1 minute.
- 4 Place on the preprogrammed thermal cycler and run the NRC HYB program. Each well contains 100  $\mu$ l.
- 5 Keep at the 58°C holding temperature for at least 90 minutes and up to 24 hours.

## Capture Hybridized Probes

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 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 Transfer all volumes to the corresponding well of a new midi plate.
- 3 Add 250 µl SMB to each well.

- 4 Shake at 1200 rpm for 5 minutes.
- 5 Incubate at room temperature for 25 minutes.
- 6 Centrifuge at  $280 \times g$  for 1 minute.
- 7 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 8 Remove and discard all supernatant from each well.
- 9 Remove from the magnetic stand.

### First Wash

- 1 Wash 2 times as follows.
  - a Add 200  $\mu\text{l}$  EWS to each well.
  - b Shake at 1800 rpm for 4 minutes.
  - c Pipette to resuspend the bead pellet further.
  - d Place on the 50°C microheating system with the lid closed for 30 minutes.
  - e Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
  - f Remove and discard all supernatant from each well.
  - g Remove 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  $\mu\text{l}$ )
  - ▶ HP3 (1.5  $\mu\text{l}$ )
- 2 Add 23  $\mu\text{l}$  elution premix to each well.
- 3 Shake at 1800 rpm for 2 minutes.
- 4 Incubate at room temperature for 2 minutes.
- 5 Centrifuge at  $280 \times g$  for 1 minute.
- 6 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 7 Transfer 21  $\mu\text{l}$  supernatant to the corresponding well of a new Hard-Shell PCR plate.
- 8 Add 4  $\mu\text{l}$  ET2 to each well.
- 9 Shake at 1200 rpm for 1 minute.
- 10 Centrifuge at  $280 \times g$  for 1 minute.

### SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 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)
- ▶ TCO (TruSight Cardio 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
TCO	-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 Add the following reagents in the order listed to each sample well.
  - ▶ RSB (15 µl)
  - ▶ EHB (50 µl)
  - ▶ TCO (10 µl)
- 2 Shake at 1200 rpm for 1 minute.
- 3 Centrifuge at 280 × g for 1 minute.
- 4 Place 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.
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 Transfer supernatant to the corresponding well of a new midi plate.
- 3 Add 250 µl SMB to each well.
- 4 Shake at 1200 rpm for 5 minutes.
- 5 Incubate at room temperature for 25 minutes.

- 6 Centrifuge at  $280 \times g$  for 1 minute.
- 7 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 8 Remove and discard all supernatant from each well.
- 9 Remove from the magnetic stand.

### Second Wash

- 1 Wash 2 times as follows.
  - a Add 200  $\mu\text{l}$  EWS to each well.
  - b Shake at 1800 rpm for 4 minutes.
  - c Pipette to resuspend the bead pellet further.
  - d Place on the  $50^{\circ}\text{C}$  microheating system with the lid closed for 30 minutes.
  - e Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
  - f Remove and discard all supernatant from each well.
  - g Remove 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  $\mu\text{l}$ )
  - ▶ HP3 (1.5  $\mu\text{l}$ )
- 2 Add 23  $\mu\text{l}$  elution premix to each well.
- 3 Shake at 1800 rpm for 2 minutes.
- 4 Incubate at room temperature for 2 minutes.
- 5 Centrifuge at  $280 \times g$  for 1 minute.
- 6 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 7 Transfer 21  $\mu\text{l}$  supernatant to the corresponding well of a new midi plate.
- 8 Add 4  $\mu\text{l}$  ET2 to each well.
- 9 Shake at 1800 rpm for 1 minute.
- 10 Centrifuge at  $280 \times g$  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 Add 45  $\mu$ l SPB to each well.
- 2 Shake at 1800 rpm for 1 minute.
- 3 Incubate at room temperature for 10 minutes.
- 4 Centrifuge at  $280 \times g$  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 2 times as follows.
  - a Add 200  $\mu$ l 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  $\mu$ l pipette to remove residual EtOH from each well.
- 9 Air-dry on the magnetic stand for 10 minutes.
- 10 Add 27.5  $\mu$ l RSB to each well.
- 11 Shake at 1800 rpm for 1 minute.
- 12 Incubate at room temperature for 2 minutes.
- 13 Centrifuge at  $280 \times g$  for 1 minute.

- 14 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 15 Transfer 25  $\mu$ l 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  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  for up to 7 days.

## Amplify Enriched Library

This step uses a 12-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 AMP12 program on the thermal cycler:
  - ▶ Choose the preheat lid option and set to 100°C
  - ▶ 98°C for 30 seconds
  - ▶ 12 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 PPC to each well.
- 2 Add 20 µl NEM to each well.
- 3 Shake at 1200 rpm for 1 minute.
- 4 Centrifuge at 280 × g for 1 minute.
- 5 Place on the preprogrammed thermal cycler and run the NEM AMP12 program. Each well contains 50 µl.

### 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  $280 \times g$  for 1 minute.
- 2 Transfer 50  $\mu$ l to the corresponding well of a new midi plate.
- 3 Add 90  $\mu$ l SPB to each well.
- 4 Shake at 1800 rpm for 1 minute.
- 5 Incubate at room temperature for 10 minutes.
- 6 Centrifuge at  $280 \times g$  for 1 minute.
- 7 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  $\mu$ l 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  $\mu$ l pipette to remove residual EtOH from each well.
- 11 Air-dry on the magnetic stand for 10 minutes.
- 12 Add 32  $\mu$ l RSB to each well.

- 13 Shake at 1800 rpm for 1 minute.
- 14 Incubate at room temperature for 2 minutes.
- 15 Centrifuge at  $280 \times g$  for 1 minute.
- 16 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- 17 Transfer 30  $\mu$ l 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  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  for up to 7 days.



## Check Enriched Libraries

Perform the following procedures to check enriched library quality.

### 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 If the library concentration is higher than the supported quantified range of the quantification method, dilute the library with RSB.
- 2 Quantify using a fluorometric method.  
Use the following formula to convert from ng/μl to nM. Assume a 400 bp library size or calculate based on the average size of the enriched library.

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

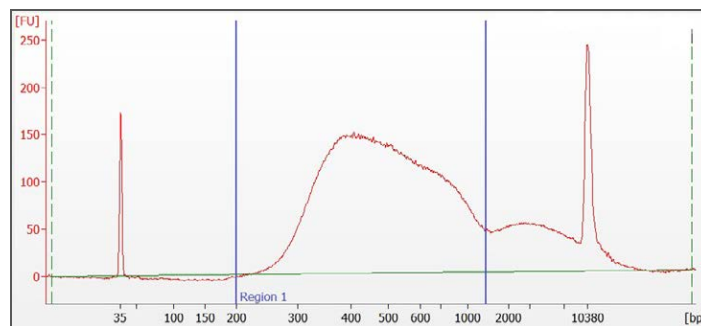
For example:

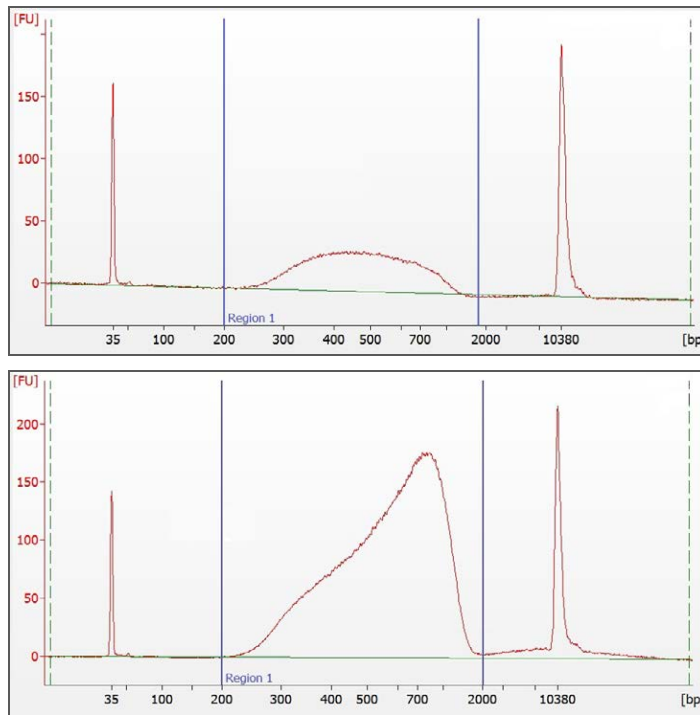
$$\frac{(15 \text{ ng/}\mu\text{l})}{(660 \text{ g/mol} * 400)} \times 10^6 = 57 \text{ nM}$$

### Assess Quality

- 1 If the library concentration is higher than the supported quantitative range for the High Sensitivity DNA chip, dilute the library 1:10 with RSB.
- 2 Run 1 μl diluted 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.

- 3 Denature and dilute pooled libraries to the loading concentration for the instrument you are using. See the denature and dilute libraries guide for your instrument.

# Supporting Information

Introduction .....	32
Acronyms .....	33
Kit Contents .....	34
Consumables and Equipment .....	37
Index Sequences .....	40



## Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all the 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
NEC1	Nextera Enriched Clean Up Plate 1
NEC2	Nextera Enriched Clean Up Plate 2
NEH1	Nextera Enrichment Hyb Plate 1
NEH2	Nextera Enrichment Hyb Plate 2
NEL	Nextera Enrichment Library Plate
NEM	Enrichment Amp Mix
NEW1	Nextera Enrichment Wash Plate 1
NEW2	Nextera Enrichment Wash Plate 2
NIL	Nextera Index Library Plate
NLA	Nextera Library Amplification Plate
NLC	Nextera Library Clean Up Plate
NLM	Library Amp Mix
NLT	Nextera Library Tagment Plate
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
ST	Stop Tagment Buffer
TCO	TruSight Cardio Oligos
TD	Tagment DNA Buffer
TDE1	Tagment DNA Enzyme TDE

## Kit Contents

Make sure that you have the reagents identified in this section before proceeding to the library preparation procedures. The TruSight Cardio Sequencing Kit is available in the following configurations.

Kit Name	Catalog #
TruSight Cardio Sequencing Kit for MiSeq, v2 chemistry (12 samples)	FC-141-1010
TruSight Cardio Sequencing Kit for NextSeq, mid-output chemistry (48 samples)	FC-141-1011

### 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 may 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 in customer's specific application.

## TruSight Cardio Sequencing Kit Contents (MiSeq, 12 samples, v2 chemistry) (FC-141-1010)

### Box 1, Store at 2°C to 8°C

Quantity	Reagent	Description
1	SPB	Sample Purification Beads
1	SMB	Streptavidin Magnetic Beads
1	ET2	Elute Target Buffer 2
1	ST	Stop Tagment Buffer

### Box 2, 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	2 N NaOH
1	PPC	PCR Primer Cocktail
1	NEM	Nextera Enrichment Amplification Mix

### Box 3, Store at -25°C to -15°C

Quantity	Description
1	Index Primer, E505
12	Index Primers, N701 to N712

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

Quantity	Description
1	TruSight Cardio Oligos

### Box 5 - PhiX Control v3, Store at -25°C to -15°C

Quantity	Description
1 tube	PhiX v3

### MiSeq Reagent Kit v2 Box 1, Store at -25°C to -15°C

Quantity	Description
1	HT1 (Hybridization Buffer)
1	MiSeq v2 Reagent Cartridge, 300 cycles

### MiSeq Reagent Kit v2 Box 2, Store at 2°C to 8°C

Quantity	Description
1	MiSeq Flow Cell
1	PR2 Bottle

## TruSight Cardio Sequencing Kit Contents (NextSeq, 48 Samples, Mid Output) (FC-141-1011)

### Box 1 - TruSight Rapid Capture Reagents, Store at 2°C to 8°C

Quantity	Reagent	Description
2	SPB	Sample Purification Beads
2	SMB	Streptavidin Magnetic Beads
1	ET2	Elute Target Buffer 2
1	ST	Stop Tagment Buffer

### Box 2 - TruSight 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 - Indexes, Store at -25°C to -15°C

Quantity	Description
4	Index Adapter, E502, E503, E505, and E506
12	Index Adapter, N701 to N712

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

Quantity	Description
4	TruSight Cardio Oligos

### Box 5 - PhiX Control v3, Store at -25°C to -15°C

Quantity	Description
1	PhiX v3

### NextSeq Reagents

Quantity	Description	Storage Temperature
1	NextSeq 500 Mid Output Reagent Cartridge v2 (300 cycles)	-25°C to -15°C
1	NextSeq 500 Mid Output Flow Cell	2°C to 8°C
1	NextSeq 500 Buffer	Room temperature
1	NextSeq Accessory Box v2	-25°C to -15°C



## 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
Tris-HCl 200 mM, pH 7 (NextSeq sequencing only)	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

Consumable	Supplier
[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 <i>Thermal Cyclers</i> on page 39.	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
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 <sup>1</sup>	Illumina, catalog # FC-130-1005
[Optional] Vacuum concentrator <sup>2</sup>	General lab supplier

<sup>1</sup> Reusable and recommended for setting up indexed adapters.

<sup>2</sup> 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

Use the following sequences when setting up a sample sheet for a MiSeq sequencing run.

Index	Sequence
E505	GTAAGGAG
N701	TAAGGCGA
N702	CGTACTAG
N703	AGGCAGAA
N704	TCCTGAGC
N705	GGACTCCT
N706	TAGGCATG
N707	CTCTCTAC
N708	CAGAGAGG
N709	GCTACGCT
N710	CGAGGCTG
N711	AAGAGGCA
N712	GTAGAGGA

Use the following sequences when setting up a NextSeq sequencing run using the BaseSpace Prep tab (available when selecting Nextera Rapid Capture under Library Prep Kit).

## Technical Assistance

For technical assistance, contact Illumina Technical Support.

**Table 1** Illumina General Contact Information

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

**Table 2** Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Japan	0800.111.5011
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
China	400.635.9898	Singapore	1.800.579.2745
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	Taiwan	00806651752
Hong Kong	800960230	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000
Italy	800.874909		

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

**Product documentation**—Available for download in PDF from the Illumina website. Go to [support.illumina.com](http://support.illumina.com), select a product, then select **Documentation & Literature**.

