TruSight™ Enrichment DNA Sample Preparation Guide

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Revision History

Part #	Revision	Date	Description of Change
15040457	B	March 2013	Edited statement regarding biomarker patents to copyright page.
15040457	A	January 2013	Initial release

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Overview

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Introduction

This protocol explains how to prepare up to 96 indexed paired-end libraries, followed by enrichment using the TruSight™ Content Set CSO (custom selected oligos), and subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina TruSight Enrichment DNA Sample Preparation Kit. The goal of this protocol is to fragment and add adapter sequences onto template DNA with a single tube TruSight Enrichment tagmentation reaction to generate multiplexed sequencing libraries that can be carried through enrichment for targeted resequencing applications.

The TruSight Enrichment DNA Sample Preparation kits are based on the Nextera Enrichment DNA Sample Prep kits and use the same reagents, but are designed to be used in conjunction with the TruSight Content Sets as the Custom Selected Oligos (CSO). The TruSight Enrichment DNA Sample Preparation protocol is the same as the Nextera Enrichment DNA Sample Prep protocol except for an increased number of cycles in the final PCR Amplification steps. To analyze the TruSight Content Sets via MiSeq Reporter Enrichment analysis flow, an Illumina provided manifest file is required. There is a unique manifest file for each TruSight Content Set. The manifest files can be downloaded from the TruSight Enrichment DNA Sample Preparation Kit Support page.

The TruSight Enrichment DNA Sample Preparation protocol offers:

Sequencing's fastest and easiest preparation

- Prep up to 12 samples in a single enrichment reaction in less than 3 hours; prep and enrichment time reduced to 2.5 days
- High manual throughput or simple automation friendly with no fragmentation bottlenecks

Lowest DNA input and excellent data quality

- Excellent data quality with low input of 50 ng
- Access precious samples with no impact to performance
- Ability to archive samples for additional analysis

Highest enrichment rates and exceptional coverage uniformity

- Efficient use of sequencing and reliable variant calling
- Reduce FTE time with the most cost-effective, high-throughput workflow

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- **Experienced User Card (EUC)** to guide you through the protocol, but with less detail than provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.**
- **Lab Tracking Form (LTF)** to record information about library preparation such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
  - Create a copy of the lab tracking form for each time you perform this protocol to prepare a library for sequencing.
  - Use it online and save it electronically or print it and fill it out manually.

NOTE
You can download the above TruSight Enrichment DNA Sample Preparation documents from the Illumina website at http://www.illumina.com. Go to the TruSight Enrichment DNA Sample Preparation support page and click the **Documentation & Literature** tab.

- The **Illumina Experiment Manager** (IEM) can be used to create your sample sheet using a wizard-based application. The sample sheet is used to record information about your samples for later use in data analysis. The IEM guides you through the steps to create your sample sheet based on the analysis workflow for your run. The IEM provides a feature for recording parameters for your sample plate, such as sample ID, single indexing, and other parameters applicable to your 96-well plate. When using IEM for sample sheet generation, make sure you select **Adapter Trimming** when you create your sample sheet for all Illumina Sequencing Platforms. Shorter inserts can lead to sequencing into the adapter, and this feature helps filter out adapter sequence from the final sequence data. The IEM can be run on any Windows platform. You can download IEM from the Illumina website at http://www.illumina.com. Go to the TruSight Enrichment DNA Sample Preparation support page and click **Downloads**. A MyIllumina account is required.
  - When prompted to select a Sample Prep Kit Type in IEM, choose **Nextera Enrichment**.
  - When creating a MiSeq compatible sample sheet in IEM, choose the **Targeted Resequencing** category and then the **Enrichment** application.

Documentation

Additional documentation is available for download from the Illumina website. Refer to the inside back cover of this guide for more information.

Getting Started

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TruSight Enrichment DNA Sample Preparation Kit

The TruSight Enrichment DNA Sample Preparation Kit is packaged in 288, 96, 48, 16, or 8 sample boxes and shipped on dry ice unless specified otherwise below.

NOTE
Certain components of the kit should be stored at a different temperature than the temperature at which they are shipped. As soon as you receive your kit, store the kit components at the specified temperature.

CAUTION
If sequencing TruSight Enrichment libraries with HiSeq2000/1000, HiScanSQ, or GAIIx, you must be sure to use the TruSeq Dual Index Sequencing Primer Boxes (Single Read or Paired End, as appropriate) for all sequencing run types: non-indexed, single-indexed, and dual-indexed. **These add-on kits are not required if sequencing a TruSight Enrichment library with the MiSeq System.**

TruSight™ Enrichment Kits

Consumable	Catalog #	*TG Catalog #
TruSight Enrichment Kit (1 Index, 8 Samples)	FC-123-1200	TG-140-1004
TruSight Enrichment Kit (2 Indices, 8 Samples)	FC-123-1201	TG-140-1001
TruSight Enrichment Kit (4 Indices, 16 Samples)	FC-123-1202	TG-140-1002
TruSight Enrichment Kit (24 Indices, 48 Samples)	FC-123-1203	TG-140-1003
TruSight Enrichment Kit (24 Indices, 96 Samples)	FC-123-1205	TG-140-1005
TruSight Enrichment Kit (96 Indices, 288 Samples)	FC-123-1206	TG-140-1006

NOTE
*TG-labeled consumables include features intended to help reduce the frequency of revalidation. They are available only under supply agreement and require you to provide a binding forecast. Please contact your account manager for more information.

NOTE
In order to support the number of samples as noted on the kit box, samples will need to be pooled appropriately prior to the enrichment step, as indicated in the user guide. For additional pooling instructions see *Library Pooling Guidelines* on page 62.

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 in order to use the product in customer's specific application.

TruSight Content Sets - Oligos Only

Consumable	*TG Catalog #
TruSight Exome Content Set	TG-141-1001
TruSight Cancer Content Set	TG-141-1002
TruSight Autism Content Set	TG-141-1003
TruSight Cardiomyopathy Content Set	TG-141-1004
TruSight Inherited Disease Content Set	TG-141-1005

NOTE
*TG-labeled consumables include features intended to help reduce the frequency of revalidation. They are available only under supply agreement and require you to provide a binding forecast. Please contact your account manager for more information.

NOTE
Illumina does not support 1-plex enrichments for the TruSight Cancer, Cardiomyopathy, or Autism Content Sets.

TruSeq Index Plate Fixture Kit

It is recommended to use the index plate fixture to assist in correctly arranging the index primers during the PCR Amplification steps. Each kit contains two fixtures and can be used for all TruSight Enrichment DNA Sample Preparation kits.

Consumable	Catalog #	TG Catalog #
TruSeq Index Plate Fixture Kit	FC-130-1005	TG-130-1005

TruSight Enrichment Kit Contents (1 Index, 8 Samples) (FC-123-1200, TG-140-1004)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
2	SMB	Streptavidin Magnetic Beads	2° to 8°C
2	WS1	Wash Solution 1	2° to 8°C
2	WS3	Wash Solution 3	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
2	WS2	Wash Solution 2	-15° to -25°C
2	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
2	ET1	Elute Target Buffer 1	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
1	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
1 tube	Index Primer, N701	-15° to -25°C

TruSight Enrichment Kit Contents (2 Indices, 8 Samples) (FC-123-1201, TG-140-1001)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	WS1	Wash Solution 1	2° to 8°C
1	WS3	Wash Solution 3	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
1	WS2	Wash Solution 2	-15° to -25°C
1	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
1	ET1	Elute Target Buffer 1	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
1	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
2 tubes	Index Primers, N701 to N702	-15° to -25°C

TruSight Enrichment Kit Contents (4 Indices, 16 Samples) (FC-123-1202, TG-140-1002)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	WS1	Wash Solution 1	2° to 8°C
1	WS3	Wash Solution 3	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
1	WS2	Wash Solution 2	-15° to -25°C
1	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
1	ET1	Elute Target Buffer 1	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
4 tubes	Index Primers, N701 to N704	-15° to -25°C

TruSight Enrichment Kit Contents (24 Indices, 48 Samples) (FC-123-1203, TG-140-1003)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	WS1	Wash Solution 1	2° to 8°C
1	WS3	Wash Solution 3	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
1	WS2	Wash Solution 2	-15° to -25°C
1	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
1	ET1	Elute Target Buffer 1	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
1	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
2	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
2 tubes	Index Primers, E501 to E502	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

TruSight Enrichment Kit Contents (24 Indices, 96 Samples) (FC-123-1205, TG-140-1005)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
2	SMB	Streptavidin Magnetic Beads	2° to 8°C
2	WS1	Wash Solution 1	2° to 8°C
2	WS3	Wash Solution 3	2° to 8°C
2	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
2	WS2	Wash Solution 2	-15° to -25°C
2	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
2	ET1	Elute Target Buffer 1	-15° to -25°C
2	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
2	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
2	TD	Tagment DNA Buffer	-15° to -25°C
3	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
4 tubes	Index Primers, E501 to E502	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

TruSight Enrichment Kit Contents (96 Indices, 288 Samples) (FC-123-1206, TG-140-1006)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
6	SMB	Streptavidin Magnetic Beads	2° to 8°C
6	WS1	Wash Solution 1	2° to 8°C
6	WS3	Wash Solution 3	2° to 8°C
6	ET2	Elute Target Buffer 2	2° to 8°C
3	ST	Stop Tagment Buffer	Room temperature

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
6	WS2	Wash Solution 2	-15° to -25°C
6	NCT1	Nextera Capture Target Buffer 1	-15° to -25°C
6	ET1	Elute Target Buffer 1	-15° to -25°C
6	HP3	2N NaOH	-15° to -25°C
3	PPC	PCR Primer Cocktail	-15° to -25°C
6	LP#-PMM	PCR Master Mix, Polymerase	-15° to -25°C
6	TD	Tagment DNA Buffer	-15° to -25°C
8	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
3	TC#-PMM	PCR Master Mix, Polymerase	-15° to -25°C

NOTE
LP#-PMM and TC#-PMM contain the same reagent, but have different volumes. They are used at different steps of the protocol, as described in this guide.

Box 3

Quantity	Reagent Name	Storage Temperature
16 tubes	Index Primers, E501 to E508	-15° to -25°C
24 tubes	Index Primers, N701 to N712	-15° to -25°C

TruSight Content Sets - Oligos Only

NOTE
Illumina does not support 1-plex enrichments for the TruSight Cancer, Cardiomyopathy, or Autism Content Sets.

TruSight Exome Content Set

Quantity	Reagent Name	Storage Temperature
1	Exome TruSight Content Set CSO	-15° to -25°C

TruSight Cancer Content Set

Quantity	Reagent Name	Storage Temperature
1	Cancer TruSight Content Set CSO	-15° to -25°C

TruSight Autism Content Set

Quantity	Reagent Name	Storage Temperature
1	Autism TruSight Content Set CSO	-15° to -25°C

TruSight Cardiomyopathy Content Set

Quantity	Reagent Name	Storage Temperature
1	Cardiomyopathy TruSight Content Set CSO	-15° to -25°C

TruSight Inherited Disease Content Set

Quantity	Reagent Name	Storage Temperature
1	Inherited Disease TruSight Content Set CSO	-15° to -25°C

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. These consumables and equipment are Illumina recommended for the TruSight Enrichment DNA Sample Preparation protocols.

Table 1 User-Supplied Consumables

Consumable	Supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 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
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
300 µl 96-well skirtless PCR plates or Twin.Tec 96-well PCR plates	E&K Scientific, part # 480096 or Eppendorf, part # 951020303
Adhesive seal roller	General lab supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Distilled water	General lab supplier

Consumable	Supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
Microseal 'A' film	BioRad, part # MSA-5001
Microseal 'B' adhesive seals	BioRad, part # MSB-1001
PCR grade water (for gel-free method)	General lab supplier
PCR tubes	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Qubit dsDNA BR Assay Kit	Life Technologies, 100 assays - catalog # Q32850 500 assays - catalog # Q32853
Qubit assay tubes or Axygen PCR-05-C tubes	Life Technologies, catalog # Q32856 or VWR, part # 10011-830
Ultra pure water	General lab supplier
Microseal 96-well PCR plates ("TCY" plate)	Bio-Rad, part # HSP-9601

Table 2 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	See table in *Thermal Cycler* section.
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA

Equipment	Supplier
[Optional] High Sensitivity Agilent DNA Chip	Agilent, part # 4067-4626
DNA Engine Multi-Bay Thermal Cycler	Bio-Rad, part # PTC-0240G or PTC-0220G, with Alpha Unit, part # ALS-1296GC
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) VWR, catalog # 14216-214 (230V)
Magnetic stand-96	Ambion, part # AM10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
Qubit 2.0 Fluorometer	Life Technologies, catalog # Q32866 http://products.invitrogen.com/ivgn/product/Q32866
Vacuum concentrator	General lab supplier
Vortexer	General lab supplier

Thermal Cycler

The following table lists the recommended settings for selected thermal cycler models. Illumina recommends that you validate any thermal cyclers not listed below if your lab has not yet performed the TruSight Enrichment DNA Sample Preparation 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

Best Practices

Adhere to the following best practices when preparing libraries for sequencing using this protocol. Several components of this kit are shipped at one temperature and stored at a warmer temperature. The components are stable at either temperature, but should be used at the warmer temperature. To avoid delay during sample preparation, each component should be stored according to the recommendations in the *Getting Started* section.

Ensuring Consistency

- **Use multichannel pipettes**—To make sure there is consistency across samples, use a multichannel pipette where possible. Calibrate pipettes periodically.
- **Pre-aliquot reagents**—To avoid unnecessary freeze-thaw cycles when performing experiments of fewer than 96 samples, Illumina recommends that you aliquot smaller volumes of reagents normally stored frozen after they are thawed for the first time.

Handling Magnetic Beads

- **Use at room temperature**—Prior to use, allow the beads to reach room temperature. Use a 25°C water bath as necessary.
- **Vortex until well-suspended**—Immediately prior to use, vortex the beads until they are well-suspended and the color appears homogeneous.
- **Allow maximum binding**—For best results, incubate your bead/sample mixtures at room temperature for the entire duration indicated in the protocol.
- **Slowly aspirate cleared solution**—After placing the plate on the magnetic stand, wait for the solution to clear before proceeding. Keep the plate on the magnetic stand when slowly aspirating cleared solution, taking care not to disturb the separated beads.

Avoiding Cross-Contamination

- **Change tips between samples**—Always use fresh pipette tips between samples and between dispensing index primers.
- **Mix plates as directed**—Mix samples with a multichannel pipette and centrifuge the plate when indicated. Do not vortex the plates.
- **Use aerosol-resistant tips**—Using aerosol-resistant pipette tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.

NOTE
If aerosol-resistant tips are not available, ensure careful pipetting to avoid contamination.

Washing with 80% Ethanol During PCR Clean-Up

- **Prepare fresh 80% ethanol**—Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting your results.
- **Remove all ethanol from wells**—Make sure that you remove all ethanol from the bottom of the wells as it might contain residual contaminants. Use a P20 multichannel pipette to remove residual ethanol and accelerate drying.
- **Allow complete evaporation**—Allow at least ten minutes of drying time on the magnetic stand at room temperature for complete evaporation. Residual ethanol can impact the performance of subsequent reactions.

Freeze/thawing for Small Number of Samples

- Each reagent tube supplied with your assay kit contains sufficient volume to process 96 samples at once, using an 8-channel pipette and a reservoir. When processing smaller sample batches (fewer than 96 samples) using a reagent reservoir, dead volume and pipetting error losses can increase. To make sure there is an accurate reagent volume for all samples, single-pipette the reagent into each well.
- To store remaining reagent, Illumina recommends freezing aliquots, rather than repeatedly freezing and thawing the supplied reagent tubes.

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- **Physically Separate Pre-PCR and Post-PCR Areas**
  - Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed (post-PCR processes).
  - Never use the same sink to wash pre-PCR and post-PCR troughs.
  - Never share the same water purification system for pre-PCR and post-PCR processes.
  - Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.
- **Use Dedicated Equipment and Supplies**
  - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
  - Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area, and then move the post-amplification reagents to the proper post-PCR storage area.

Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).

CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.

Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:

- Bench tops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps achieve this.

Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Weekly Cleaning of All Lab Areas

Once a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).

- Clean all bench tops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.

- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.
- Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Acronyms

Table 3 TruSight Enrichment DNA Sample Preparation Acronyms

Acronym	Definition
CAP	Cleaned Amplification Plate
CSO	Custom Selected Oligos
CTP1	Capture Target Plate 1
CTP2	Capture Target Plate 2
dsDNA	Double-stranded DNA
ET1	Elute Target Buffer 1
ET2	Elute Target Buffer 2
HP3	2N NaOH
IWP1	Intermediate Wash Plate 1
IWP2	Intermediate Wash Plate 2
NCT1	Nextera Capture Target Buffer 1
NEA1	Nextera Enrichment Amplification Plate 1
NEA2	Nextera Enrichment Amplification Plate 2
NEL	Nextera Enrichment Library Plate
NET1	Nextera Enrichment Tagmentation Plate 1
NET2	Nextera Enrichment Tagmentation Plate 2
PCR	Polymerase Chain Reaction
PMM	PCR Master Mix, Polymerase

Acronym	Definition
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
ST	Stop Tagment Buffer
SMB	Streptavidin Magnetic Beads
TAP1	Target Amplification Plate 1
TD	Tagment DNA Buffer
TDE1	Tagment DNA Enzyme
TTP	Temporary Target Plate
WS1	Wash Solution 1
WS2	Wash Solution 2
WS3	Wash Solution 3
WTP1	Wash Target Plate 1
WTP2	Wash Target Plate 2

DNA Input Recommendations

The TruSight Enrichment DNA Sample Preparation Kit protocol is optimized for 50 ng of genomic DNA total. Illumina strongly recommends quantifying the starting genomic material.

Input DNA Quantitation

TruSight Enrichment DNA Sample Preparation library preps use an enzymatic DNA fragmentation step and thus can be more sensitive to DNA input compared to mechanical fragmentation methods. The ultimate success of enrichment strongly depends on using an accurately quantified amount of input DNA library. Therefore, the correct quantitation of the DNA library is essential.

Assessing DNA Quality

Absorbance measurements at 260 nm are commonly used to quantify DNA. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. This protocol is optimized for DNA with absorbance ratio values of 1.8–2.0.

TruSight Enrichment Protocol

Chapter 3

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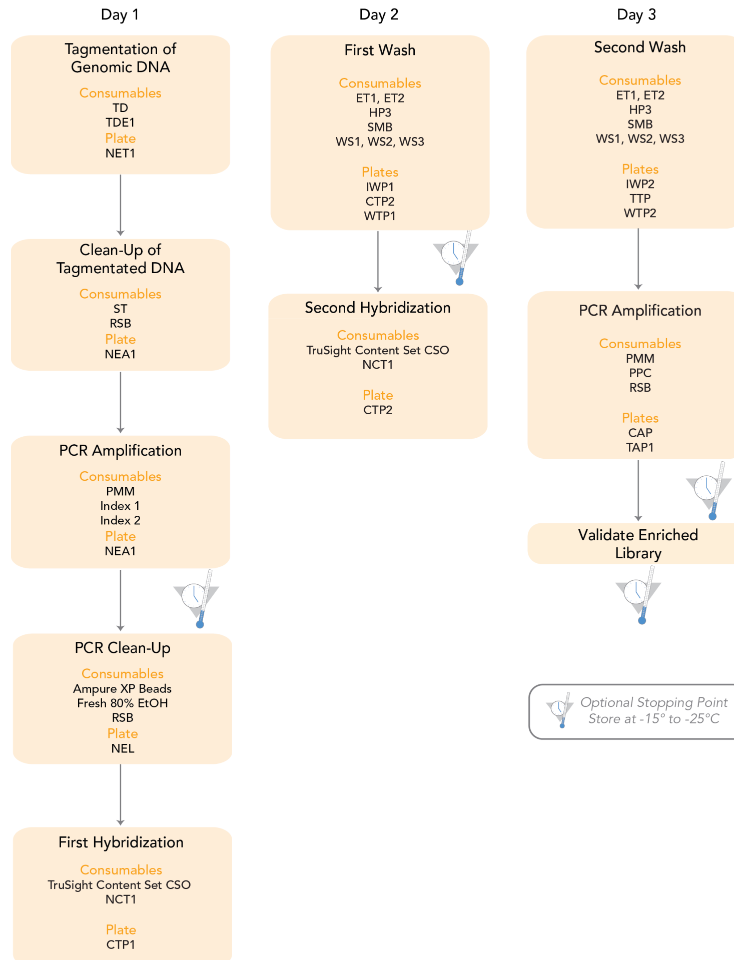
TruSight Enrichment Workflow

The following diagram illustrates the workflow using the TruSight Enrichment DNA Sample Preparation Kit. Safe stopping points are marked between steps.

Figure 1 TruSight Enrichment DNA Sample Preparation Workflow (For 8 samples)



Tagmentation of Genomic DNA

During this step genomic DNA is tagmented (tagged and fragmented) by the Nextera transposome. The Nextera transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps.

Consumables

Item	Quantity	Storage	Supplied By
TD (Tagment DNA Buffer)	1 tube	-15° to -25°C	Illumina
TDE1 (Tagment DNA Enzyme)	1 tube	-15° to -25°C	Illumina
96-well hard shell TCY plate	1 plate	Room temperature	User
Genomic DNA (2.5 ng/µl)	50 ng	-15° to -25°C	User
Microseal 'B' adhesive seal		Room temperature	User

Preparation

1. Remove the TD, TDE1, and genomic DNA from -15° to -25°C storage and thaw on ice.

NOTE
In preparation for the next step of the protocol, ensure ST buffer is at room temperature, and there are no particles or precipitate visible in the solution.

2. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.

Procedure

NOTE
Ensure the reaction is assembled in the order described for optimal kit performance. The reaction does not need to be assembled on ice.

1. Label a new 96-well TCY plate "NET1" (Nextera Enrichment Tagmentation Plate 1) with a smudge resistant pen.

2 Add 20 µl of genomic DNA at 2.5 ng/µl (50 ng total) to each sample well of the NET1 plate.

3 Add 25 µl of TD Buffer to the wells containing genomic DNA. Change tips between samples.

NOTE
Calculate the total volume of TD for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NET1 plate.

4 Add 5 µl of TDE1 to the wells containing genomic DNA and TD Buffer. Change tips between samples.

NOTE
Calculate the total volume of TDE1 for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NET1 plate.

5 Using a multichannel pipette, gently pipette up and down 10 times to mix. Change tips between samples.

6 Cover the NET1 plate with Microseal 'B'.

7 Centrifuge at 280 xg at 20°C for 1 minute.

8 Place the NET1 plate in a thermocycler and run the following program:

NOTE
Ensure that the thermocycler lid is heated during the incubation.

- 55°C for 5 minutes
- Hold at 10°C

9 Proceed to *Clean-Up of Tagmented DNA*.

Clean-Up of Tagmented DNA

The tagmented DNA is purified from the Nextera transposome. This step is critical because the Nextera transposome can bind tightly to DNA ends and will interfere with downstream processes if not removed.

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
ST Buffer	1 tube	Room temperature	Illumina
AMPure XP Beads	1 tube	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)			User
96-well hard shell TCY plate	1 plate		User
96-well MIDI plate	1 plate		User

Preparation

1. Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
2. Remove the NET1 plate from the Thermocycler and remove the seal.
3. Visually inspect the ST buffer to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.

Procedure

1. Add 15 µl ST buffer to each well of the NET1 plate with sample.
2. Gently pipette up and down 10 times to mix.
3. Incubate at room temperature (20° to 25°C) for 5 minutes.

4 Centrifuge the NET1 plate at 280 xg at 20°C for 1 minute.

5 Using a multichannel P200 transfer the entire solution in each well of the NET1 plate to a clean 96-well midi plate labeled "NET2" (Nextera Enrichment Tagmentation Plate 2) with a smudge resistant pen.

6 Add 52 µl of well-resuspended AMPure XP beads to each well of the NET2 plate with sample and gently pipette up and down 10 times.

7 Incubate at room temperature for 10 minutes.

8 Cover the NET2 plate with a seal and centrifuge at 280 xg at 20°C for 1 minute.

9 Remove the seal and place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

10 Remove and discard supernatant.

11 While the NET2 plate is still on the magnet slowly add 200 µl of freshly made 80% ethanol to each sample. Wait 30 seconds.

12 Remove and discard the ethanol.

13 Repeat steps 11 and 12. Make sure there is no residual ethanol left in the plate.

14 While the NET2 plate is still on the magnet stand dry beads at room temperature for 10 minutes.

15 Remove the NET2 plate from the magnet and add 22.5 µl of RSB buffer.

16 Pipette mix up and down 10 times to resuspend beads.

17 Place the NET2 plate on the magnet for 2 minutes until the solution becomes clear.

18 Label a new 96-well TCY plate "NEA1" (Nextera Enrichment Amplification Plate 1) with a smudge resistant pen.

19 Transfer 20 µl of supernatant to the new NEA1 plate.

NOTE
(Optional) Check the products of the tagmentation reaction by loading 1 µl of supernatant on a HS Bioanalyzer chip. This should produce a broad distribution of DNA fragments with a size range from ~150 bp – <1 Kb.

PCR Amplification

In this step, the purified tagmented DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) and sequencing, as well as common adapters (P5 and P7) required for cluster generation and sequencing. It is critical to use the full amount of recommended input DNA, as well as to not add extra cycles of PCR cycles to ensure high quality libraries that produce high-quality sequencing results.

Consumables

Item	Quantity	Storage	Supplied By
PMM (PCR Master Mix)	1 tube	-15° to -25°C	Illumina
Index 1 primers (N7XX)	1 tube each index	-15° to -25°C	Illumina
Index 2 primers (E5XX)	1 tube each index	-15° to -25°C	Illumina
TruSeq Index Plate Fixture			Illumina
Microseal 'A' adhesive film			User
96-well hard shell TCY plate	1 plate		User

NOTE
The TruSight Enrichment DNA Sample Preparation kit is designed to work only with Index 2 primers with the "E" prefix. Index 2 primers from other Nextera sample prep kits should not be used.

Procedure

NOTE
For pooling libraries prior to enrichment, it is recommended to pool libraries so all Index 1 (i7) indexes are unique. Choose Index 1 and index 2 primers for PCR accordingly. For further details refer to Appendix A of the *TruSight Enrichment DNA Sample Preparation Guide*.

NOTE
PCR Set up should be done in the NEA1 plate, same plate that your final elution is.

1 To each well of the NEA1 plate containing sample add:

- 20 µl of PMM
- 5 µl of the Index 1 (i7, N7xx) primer
- 5 µl of the Index 2 (i5, E5xx) primer
- Total volume = 50 µl

2 Gently pipette mix up and down.

3 Cover the NEA1 plate with Microseal 'A' and seal with a rubber roller.

4 Centrifuge at 280 xg at 20°C for 1 minute.

5 Perform PCR using the following program on a thermal cycler:

NOTE
Ensure that the thermocycler lid is heated during the incubation. Pre-heat lid 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

SAFE STOPPING POINT
If you do not plan to immediately proceed to *PCR Clean-Up* following the completion of PCR, the NEA1 plate can remain on the thermalcycler overnight, or you can store it at 2° to 8°C up to two days.

PCR Clean-Up

This step uses AMPure XP beads to purify the library DNA, and provides a size selection step that removes very short library fragments from the population.

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
AMPure XP beads		2° to 8°C	User
80% Ethanol, freshlyprepared			User
96-well MIDI plates	1 plate		User
96-well TCY plates	1 plate		User

Preparation

1 Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.

Procedure

1 Remove the NEA1 plate from the thermocycler and centrifuge at 280 xg at 20°C for 1 minute.

2 Carefully remove the cover so the solution does not splash (if this happens centrifuge again).

3 Label a new 96-well TCY plate "NEA2" (Nextera Enrichment Amplification Plate 2) with a smudge resistant pen.

4 Transfer the entire PCR reaction to the NEA2 plate.

5 Add 45 µl of well-resuspended AMPure XP beads to each well of the NEA2 plate with sample.

6 Gently pipette up and down 10 times to mix.

7. Incubate at room temperature for 10 minutes.
8. Place the NEA2 plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
9. Carefully remove and discard the supernatant.
10. While the NEA2 plate is still on the magnet slowly add 200 µl of freshly made 80% ethanol to each sample and wait 30 seconds.
11. Remove and discard the entire ethanol wash.
12. Repeat steps 10 and 11.
13. While the NEA2 plate is still on the magnet stand dry beads at room temperature for 15 minutes.
14. Remove the NEA2 plate from the magnet and add 40 µl of RSB buffer.
15. Pipette mix up and down 10 times to resuspend beads.
16. Place the NEA2 plate on the magnet for 2 minutes until the solution becomes clear.
17. Label a new 96-well TCY plate "NEL" (Nextera Enrichment Library Plate) with a smudge resistant pen.
18. Transfer 38 µl of supernatant to the NEL plate.
19. Quantify the library by either Picogreen or Qubit.

First Hybridization

This process mixes the DNA library with capture probes of targeted regions. The recommended hybridization time makes sure that targeted regions bind to the capture probes thoroughly. It also describes how to combine multiple libraries with different indices into a single pool prior to enrichment.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
NCT1 (Nextera Capture Target Buffer 1)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	1 plate		User
Microseal 'B' adhesive seal			User
PCR grade water			User
500 ng DNA library from NEL Plate			User

Preparation

- Remove the CSO tube from -15° to -25°C storage and thaw at room temperature.
- Remove the NCT1 tube from -15° to -25°C storage and thaw at room temperature.
- Pre-program the thermal cycler as follows:
  - 95°C for 10 minutes
  - 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle
  - 58°C for forever
- Pre-heat the thermal cycler lid to 100°C.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CTP1" (Capture Target Plate 1) with a smudge resistant pen.

Pool Libraries

NOTE

In order to support the number of samples as noted on the kit box, samples will need to be pooled appropriately prior to the enrichment step, as indicated in the user guide. For additional pooling instructions see *Library Pooling Guidelines* on page 62.

1. Reference Table 4 for the amount of DNA libraries to use for enrichment. Illumina recommends using 500 ng of each DNA library, quantified by picogreen or the Qubit Fluorometric Quantitation system. See *DNA Input Recommendations* on page 25. If pooling libraries, combine 500 ng of each DNA library. If the total volume is greater than 40 µl, use a vacuum concentrator without heat to reduce the pooled sample volume to 40 µl.
2. The recommended pre-enrichment pooling strategy is to pool up to 12 libraries each with a unique Index 1/i7 index. With this pooling approach samples can be sequenced using a single index read workflow, as described in the HiSeq and GAIIx user guides. If Index1/i7 indexes are not unique, ensure that libraries with different Index 2/i5 indexes are included (e.g. N703/E501 and N703/E502). With this approach, samples can be sequenced using a dual index read workflow, as described in the HiSeq and GAIIx user guides. Please see *Library Pooling Guidelines* on page 62 for further details.

NOTE
Adding >500 ng library DNA into the enrichment assay (up to 1 ug per sample) may produce greater mean coverage/sample.

Table 4 DNA Libraries for Enrichment

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

Make CTP1

1. Thoroughly vortex the NCT1 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the NCT1 tube until it appears clear.

2. In the order listed below, prepare the reaction mix in each well of the new 300 µl 96-well PCR plate or twin.tech 96-well PCR plate labeled CTP1. Gently pipette the entire volume up and down 5–10 times to mix thoroughly. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
DNA library from NEL plate	40
NCT1	50
TruSight Content Set CSO	10
Total Volume per Sample	**100**

3. Seal the CTP1 plate with a Microseal 'B' adhesive seal. Make sure that the plate is tightly sealed to prevent potential evaporation. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

Incubate CTP1

1. Centrifuge the CTP1 plate at 280 xg at 20°C for 1 minute.
2. Place the sealed CTP1 plate on the pre-programmed thermal cycler. Close the lid and incubate as follows:
   a. Choose the pre-heat lid option and set to 100°C.
   b. 95°C for 10 minutes.
   c. 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
   d. 58°C for 16–20 hours.

First Wash

This process uses streptavidin beads to capture probes containing the targeted regions of interest. Three wash steps remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second hybridization.

Consumables

Item	Quantity	Storage	Supplied By
HP3 (2N NaOH)	1 tube	-15° to -25°C	Illumina
ET1 (Elute Target Buffer 1)	1 tube	-15° to -25°C	Illumina
ET2 (Elute Target Buffer 2)	1 tube	2° to 8°C	Illumina
SMB (Streptavidin Magnetic Beads)	1 tube	2° to 8°C	Illumina
WS1 (Wash Solution 1)	1 tube	2° to 8°C	Illumina
WS2 (Wash Solution 2)	1 tube	-15° to -25°C	Illumina
WS3 (Wash Solution 3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate			User
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
Microseal 'B' adhesive seal	5		User
PCR grade water			User
PCR tubes			User

Preparation

- Remove the SMB, ET2, WS1, and WS3 tubes from 2° to 8°C storage and let stand at room temperature.

- Remove the ET1, HP3, and WS2 tubes from -15° to -25°C storage and thaw at room temperature.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "IWP1" (Intermediate Wash Plate 1) with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CTP2" (Capture Target Plate 2) with a smudge resistant pen.
- Label a new 96-well MIDI plate "WTP1" (Wash Target Plate 1) with a smudge resistant pen.

Make WTP1

1. Remove the CTP1 plate from the thermal cycler.
2. Centrifuge the CTP1 plate at 280 xg at 20°C for 1 minute.
3. Place the CTP1 plate on a 96-well rack and remove the adhesive seal from the plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents of each well from the CTP1 plate to the corresponding well of the new 96-well MIDI plate labeled WTP1.

NOTE
It is normal to see a small degree of sample loss after overnight hybridization. However, if the sample loss is greater than 15%, Illumina does not recommended proceeding with the sample preparation. This amount of loss can be caused by poor sealing or not heating the lid.

5. Vortex the SMB tube until the beads are well dispersed, then add 250 µl of well-mixed SMB to the wells of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times until mixed.
6. Seal the WTP1 plate with a Microseal 'B' adhesive seal.
7. Let the WTP1 plate stand at room temperature for 30 minutes.
8. Centrifuge the WTP1 plate at 280 xg at 20°C for 1 minute.
9. Remove the adhesive seal from the WTP1 plate.
10. Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.
11. Remove and discard all of the supernatant from each well.
12. Remove the WTP1 plate from the magnetic stand.

Wash 1 WTP1 and Wash 2 WTP1

Perform WS1 Clean Up and WS2 Clean Up on the WTP1 plate as follows:

WS1 Clean Up

1 Thoroughly vortex the WS1 tube. Visually make sure that no crystal structures are present.

NOTE
If crystals are observed, vortex the WS1 tube until no crystal structures are visible.

2 Add 200 µl WS1 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times to make sure the beads are fully resuspended.

3 Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Remove the WTP1 plate from the magnetic stand.

WS2 Clean Up

1 Thoroughly vortex the WS2 tube. Visually make sure that the WS2 is mixed thoroughly.

2 Add 200 µl WS2 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

3 Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Remove the WTP1 plate from the magnetic stand.

6 Add 200 µl WS2 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

7 Transfer the entire contents of each well of the WTP1 plate to the corresponding well of the new 96-well PCR plate labeled IWP1.

8 Seal the IWP1 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

9 Incubate the IWP1 plate on the thermal cycler at 42°C for 30 minutes with a heated lid set to 100°C.

NOTE
For optimal results, it is important that the thermal cycler lid be heated to 100°C.

10 Place the magnetic stand next to the thermal cycler for immediate access.

11 Remove the IWP1 plate from the thermal cycler and ***immediately*** place it on the magnetic stand for 2 minutes until the liquid appears clear.

12 Remove the adhesive seal from the IWP1 plate.

13 Immediately remove and discard all of the supernatant from each well.

14 Remove the IWP1 plate from the magnetic stand.

15 Add 200 µl WS2 to each sample well of the IWP1 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

16 Repeat steps 8–13 once.

Wash 3 WTP1

Perform WS3 Clean Up and Elute Target on the WTP1 plate as follows:

WS3 Clean Up

1 Remove the IWP1 plate from the magnetic stand.

2 Add 200 µl WS3 to each well of the IWP1 plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.

3 Place the IWP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Repeat steps 1–4 once.

6 To remove any residual WS3, seal the IWP1 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.
7 Briefly centrifuge the IWP1 plate to collect any residual WS3.
8 Place the IWP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.
9 Carefully remove the adhesive seal from the IWP1 plate to avoid spilling the contents of the wells.
10 Remove and discard any residual supernatant from each well.

Elute Target

1 Mix the following reagents in the order listed in a separate PCR tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
ET1	28.5
HP3	1.5
Total Volume per Sample	**30**

2 Remove the IWP1 plate from the magnetic stand.
3 Add 23 µl of the elution pre-mix to each well of the IWP1 plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Make sure that the beads are fully resuspended.
4 Seal the IWP1 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.
5 Let the IWP1 plate stand at room temperature for 5 minutes.
6 Centrifuge the IWP1 plate at 280 xg at room temperature for 1 minute.
7 Place the IWP1 plate on the magnetic stand for 2 minutes until the liquid appears clear.
8 Carefully remove the adhesive seal from the IWP1 plate to avoid spilling the contents of the wells.
9 Transfer 21 µl of supernatant from each well of the IWP1 plate to the corresponding well of the new 96-well PCR plate labeled CTP2.

10 Add 4 µl ET2 to each well of the CTP2 plate containing samples to neutralize the elution. Gently pipette the entire volume up and down 5–10 times to mix thoroughly.

11 Seal the CTP2 plate with Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

12 Store the remaining reagents as follows:
   a Place the SMB, ET2, WS1, and WS3 tubes in 2° to 8°C storage.
   b Place the ET1, HP3, and WS2 tubes in -15° to -25°C storage.
   c Discard any remaining elution pre-mix.

SAFE STOPPING POINT
If you do not plan to proceed to *Second Hybridization* on page 47 immediately, the protocol can be safely stopped here. If you are stopping, seal the CTP2 plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days. When proceeding, thaw the CTP2 plate on ice.

Second Hybridization

This process mixes the first elution of the DNA library with the capture probes of target regions. The second hybridization make sure that the targeted regions are further enriched.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
NCT1 (Nextera Capture Target Buffer 1)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	1 plate		User
Microseal 'B' adhesive seal			User
PCR grade water			User

Preparation

- Remove the CSO tube from -15° to -25°C storage and thaw at room temperature.
- Remove the NCT1 tube from -15° to -25°C storage and thaw at room temperature.
- Remove the CTP2 plate from -15° to -25°C storage, if it was stored at the conclusion of *First Wash* on page 41 and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed CTP2 plate to 280 xg for 1 minute
  - Remove the adhesive seal from the thawed CTP2 plate.
- Pre-program the thermal cycler as follows:
  a. 95°C for 10 minutes.
  b. 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
  c. 58°C for forever.
- Pre-heat the thermal cycler lid to 100°C.
- Ensure CTP2 plate is completely thawed. Centrifuge at 280 xg for 1 minute.

Add CTO/CSO

NOTE
The second hybridization should be set up in the same plate that the elution of the first wash is in, CTP2.

1 Thoroughly vortex the NCT1 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the NCT1 tube until it appears clear.

2 In the order listed below, add the following to each well of the CTP2 plate. Gently pipette the entire volume up and down 5-10 times to mix thoroughly. Multiply each volume by the number of samples being prepared.

Reagent	Volume (µl)
NCT1	50
TruSight Content Set CSO	10
PCR Grade Water	15
First elution	25
Total Volume per Sample	**100**

3 Seal the CTP2 plate with a Microseal ‘B’ adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

Incubate CTP2

1 Centrifuge the CTP2 plate at 280 xg at 20°C for 1 minute.

2 Place the sealed CTP2 plate on the pre-programmed thermal cycler. Close the lid and incubate as follows:

  a Choose the pre-heat lid option and set to 100°C.
  b 95°C for 10 minutes.
  c 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
  d 58°C for 16–20 hours.

Second Wash

This process uses streptavidin beads to capture probes containing the targeted regions of interest. Three wash steps remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

NOTE
These procedures are similar to the *First Wash on page 41*.

Consumables

Item	Quantity	Storage	Supplied By
HP3 (2N NaOH)	1 tube	-15° to -25°C	Illumina
ET1 (Elute Target Buffer 1)	1 tube	-15° to -25°C	Illumina
ET2 (Elute Target Buffer 2)	1 tube	2° to 8°C	Illumina
SMB (Streptavidin Magnetic Beads)	1 tube	2° to 8°C	Illumina
WS1 (Wash Solution 1)	1 tube	2° to 8°C	Illumina
WS2 (Wash Solution 2)	1 tube	-15° to -25°C	Illumina
WS3 (Wash Solution 3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate			User
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
Microseal 'B' adhesive seal	5		User
PCR grade water			User
PCR tubes			User

Preparation

- Remove the SMB, ET2, WS1, and WS 3 tubes from 2° to 8°C storage and let stand at room temperature.
- Remove the ET1, HP3, and WS2 tubes from -15° to -25°C storage and thaw at room temperature.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "IWP2" (Intermediate Wash Plate 2) with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "TTP" (Temporary Target Plate) with a smudge resistant pen.
- Label a new 96-well MIDI plate "WTP2" (Wash Target Plate 2) with a smudge resistant pen.
- [Optional] Label one new PCR tube per sample “Second Elution for qPCR”.

Make WTP2

1. Remove the CTP2 plate from the thermal cycler.
2. Centrifuge the room temperature CTP2 plate at 280 xg at room temperature for 1 minute.
3. Place the CTP2 plate on a 96-well rack and remove the adhesive seal from the plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents from each well of the CTP2 plate to the corresponding well of the new 96-well MIDI plate labeled WTP2.

NOTE
It is normal to see a small degree of sample loss after overnight hybridization. However, if the sample loss is greater than 15%, Illumina does not recommended proceeding with the sample preparation. This amount of loss can be caused by poor sealing or not heating the lid.

5. Vortex the SMB tube until the beads are well dispersed, then add 250 µl of well-mixed SMB to the wells of the WTP2 plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
6. Seal the WTP2 plate with a Microseal ‘B’ adhesive seal.
7. Let the WTP2 plate stand at room temperature for 30 minutes.
8. Centrifuge the WTP2 plate at 280 xg at 20°C for 1 minute.

9 Remove the adhesive seal from the WTP2 plate.

10 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

11 Remove and discard all of the supernatant from each well.

12 Remove the WTP2 plate from the magnetic stand.

Wash 1 WTP2 and Wash 2 WTP2

Perform WS1 Clean Up and WS2 Clean Up on the WTP2 plate as follows:

WS1 Clean Up

1 Thoroughly vortex the WS1 tube. Visually make sure that no crystal structures are present.

NOTE
If crystals are observed, vortex the WS1 tube until no crystal structures are visible.

2 Add 200 µl WS1 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times to make sure the beads are fully resuspended.

3 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Remove the WTP2 plate from the magnetic stand.

WS2 Clean Up

1 Thoroughly vortex the WS2 tube. Visually make sure that the WS2 is mixed thoroughly.

2 Add 200 µl WS2 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

3 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Remove the WTP2 plate from the magnetic stand.

6 Add 200 µl WS2 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

7 Transfer the entire contents of each well of the WTP2 plate to the corresponding well of the new 96-well PCR plate labeled IWP2.

8 Seal the IWP2 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

9 Incubate the IWP2 plate on the thermal cycler at 42°C for 30 minutes with a heated lid set to 100°C.

NOTE
For optimal results, it is important that the thermal cycler lid be heated to 100°C.

10 Place the magnetic stand next to the thermal cycler for immediate access.

11 Remove the IWP2 plate from the thermal cycler and ***immediately*** place it on the magnetic stand for 2 minutes until the liquid appears clear.

12 Remove the adhesive seal from the IWP2 plate.

13 Immediately remove and discard all of the supernatant from each well.

14 Remove the IWP2 plate from the magnetic stand.

15 Add 200 µl WS2 to each sample well of the IWP2 plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Make sure that the beads are fully resuspended.

16 Repeat steps 8–13 once.

Wash 3 WTP2

Perform WS3 Clean Up and Elute Target on the WTP2 plate as follows:

WS3 Clean Up

1 Remove the IWP2 plate from the magnetic stand.

2 Add 200 µl WS3 to each well of the IWP2 plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.

3 Place the IWP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

4 Remove and discard all of the supernatant from each well.

5 Repeat steps 1–4 once.

6 To remove any residual WS3, seal the IWP2 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

7 Briefly centrifuge the IWP2 plate to collect any residual WS3.

8 Place the IWP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

9 Carefully remove the adhesive seal from the IWP2 plate to avoid spilling the contents of the wells.

10 Remove and discard any residual supernatant from each well.

Elute Target

1 Mix the following reagents in the order listed in a separate PCR tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
ET1	28.5
HP3	1.5
Total Volume per Sample	**30**

2 Remove the IWP2 plate from the magnetic stand.

3 Add 23 µl of the elution pre-mix to each well of the IWP2 plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Make sure that the beads are fully resuspended.

4 Seal the IWP2 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

5 Let the IWP2 plate stand at room temperature for 5 minutes.

6 Centrifuge the IWP2 plate at 280 xg at 20°C for 1 minute.

7 Place the IWP2 plate on the magnetic stand for 2 minutes until the liquid appears clear.

8. Carefully remove the adhesive seal from the IWP2 plate to avoid spilling the contents of the wells.
9. Transfer 21 µl of supernatant from each well of the IWP2 plate to the corresponding well of the new 96-well PCR plate labeled TTP.
10. Add 4 µl ET2 to each well of the TTP plate containing samples to neutralize the elution. Gently pipette the entire volume of each well up and down 10-20 times to mix thoroughly.
11. [Optional] The Second Elution for qPCR tube can be used for yield quantification. To do so, dilute 2 µl of supernatant from each well of the TTP plate in 98 µl PCR grade water (1:50 dilution) in a new PCR tube labeled "Second Elution for qPCR". Cap each tube and store at -15° to -25°C.
12. Store the remaining reagents as follows:
    a. Place the SMB, ET2, WS1, and WS3 tubes in 2° to 8°C storage.
    b. Place the ET1, HP3, and WS2 tubes in -15° to -25°C storage.
    c. Discard any remaining elution pre-mix.

PCR Amplification

This process uses PCR to amplify the enriched DNA library for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
PMM (PCR Master Mix)	1 tube	-15° to -25°C	Illumina
PPC (PCR Primer Cocktail)	1 tube	-15° to -25°C	Illumina
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
AMPure XP beads		2° to 8°C	User
80% Ethanol, freshly-prepared			User
Microseal 'B' adhesive seal	3		User

Preparation

- Remove one tube each of PMM and PPC from -15° to -25°C storage and thaw on ice.

NOTE
If you do not intend to consume the PMM and PPC reagents in one use, dispense the reagents into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Briefly centrifuge the thawed PPC and PMM tubes for 5 seconds.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-program the thermal cycler as follows:
  - Choose the pre-heat 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

- Label a 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CAP" (Cleaned Amplification Plate).
- Label a 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "TAP1" (Target Amplification Plate 1).

Add PPC

1 Add the following to each well of the new TAP1 300 µl 96-well PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Reagent	Volume (µl)
Second Elution from TTP plate	20
PMM	25
PPC	5
Total Volume per Sample	**50**

2 Seal the TAP1 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

3 Centrifuge the TAP1 plate at 280 xg at 20°C for 1 minute.

Amp PCR

1 Place the sealed TAP1 plate on the pre-programmed thermal cycler. Close the lid and incubate using the pre-programmed settings:

a Choose the pre-heat lid option and set to 100°C.

b 98°C for 30 seconds.

c 12 cycles of:

- 98°C for 10 seconds
- 60°C for 30 seconds
- 72°C for 30 seconds

d 72°C for 5 minutes.

e Hold at 10°C.

Make CAP

1. Remove the adhesive seal from the TAP1 plate.
2. Vortex the AMPure XP Beads until the beads are well dispersed, then add 90 µl of the mixed AMPure XP Beads to each well of the TAP1 plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Incubate the TAP1 plate at room temperature for 15 minutes.
4. Place the TAP1 plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
5. Using a 200 µl single or multichannel pipette, remove and discard 140 µl of the supernatant from each well of the TAP1 plate.

NOTE
Leave the TAP1 plate on the magnetic stand while performing the following 80% EtOH wash steps (6–8).

6. With the TAP1 plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.
7. Incubate the TAP1 plate for at least 30 seconds at room temperature, then remove and discard the supernatant from each well.
8. Repeat steps 6–7 once for a total of two 80% EtOH washes.
9. Keep the TAP1 plate on the magnetic stand and allow plate to stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.
10. Resuspend the dried pellet in each well with 30 µl RSB. Gently pipette the entire volume up and down 10 times to mix thoroughly.
11. Incubate the TAP1 plate at room temperature for 2 minutes.
12. Place the TAP1 plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
13. Transfer 28 µl of the clear supernatant from each well of the TAP1 plate to the corresponding well of the new 96-well PCR plate labeled CAP.

SAFE STOPPING POINT

If you do not plan to proceed to *Enriched Library Validation* on page 59 immediately, the protocol can be safely stopped here. If you are stopping, seal the CAP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Enriched Library Validation

Illumina recommends performing the following procedure for quality control analysis on your enriched DNA library and quantification of the DNA library templates.

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of enriched DNA library templates. Quantitate libraries using qPCR as described in the Illumina *Sequencing Library qPCR Quantification Guide*.

References

Library Pooling Guidelines 62

Library Pooling Guidelines

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel need to be read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. Follow the instructions described here to determine which libraries are pooled pre-enrichment.

The TruSight Enrichment DNA Sample Preparation kit supports pre-enrichment pooling of up to 12 different indexed samples. For pooling of 12 samples pre-enrichment, the recommended strategy is to pool samples with Index1/i7 701-712 (any Index2/i5), followed by a single-index sequencing run. For pooling <12 samples the recommended strategy is to set up a single index workflow sequencing run using different Index1/i7 indexes (any Index2/i5). Reference Table 5 to ensure proper index combinations. See Table 6 for pooling details when using both Index1/i7 and Index2/i5, followed by a dual indexed sequencing run.

NOTE
In order to support the number of samples as noted on the kit box, samples will need to be pooled appropriately prior to the enrichment step, as indicated in the user guide.

Table 5 Libraries Pooled: 6 or Fewer; Sequencing Workflow: Single Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	• [option 1] N702 and N701 • [option 2] N702 and N704	
3-plex	• [option 1] N701, N702, and N704 • [option 2] N703, N705, and N706	
4- or 5-plex	• [option 1] N701, N702, N704, and any other Index 1 adapter • [option 2] N703, N705, N706, and any other Index 1 adapter	
6-plex	N701, N702, N703, N704, N705, and N706	

Table 6 Sequencing Workflow: Single or Dual Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, Dual Index	• [option 1] N701, N702, N704, and any other Index 1 adapter (as needed) • [option 2] N703, N705, N706, and any other Index 1 adapter (as needed)	• [option 1] E501 and E502 • [option 2] E503 and E504 • [option 3] E505 and E506
7–12 plex, Single Index	• N701–N706 and any other Index 1 adapter (as needed)	• Any Index 2 (i5) adapter

The table below represents only some of the acceptable combinations. Alternatively, please check the real sequences of each index in Table 6 to make sure each base position will have signal in both color channels for the index read:

Good				Bad			
Index 1		Index 2		Index 1		Index 2	
705	GGACTCCT	503	TATCCTCT	705	GGACTCCT	502	CTCTCTAT
706	TAGGCATG	503	TATCCTCT	706	TAGGCATG	502	CTCTCTAT
701	TAAGGCGA	504	AGAGTAGA	701	TAAGGCGA	503	TATCCTCT
702	CGTACTAG	504	AGAGTAGA	702	CGTACTAG	503	TATCCTCT
	√√√√√√√√		√√√√√√√√		√√√√√√√√		√√√√xxxx

√=signal in both color
x=signal missing in one color channel

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 7 Illumina General Contact Information

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

Table 8 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Additional product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

Illumina
San Diego, California, U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com