TruSeq® Enrichment Guide

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ILLUMINA PROPRIETARY
Part # 15013230 Rev. J
July 2012

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Introduction

This protocol explains how to capture exome or custom sequences from a human DNA library that was prepared using an Illumina® TruSeq® DNA Sample Preparation kit. Reagents provided in the TruSeq Exome Enrichment or TruSeq Custom Enrichment Kits subsequently prepare the library for sequencing targeted regions on the Illumina sequencing platform. The goal of this protocol is to enrich for sequences in a solution using two rounds of hybridizations.

The enrichment protocol offers:

- Simple and scalable workflow
  - **For Exome Enrichment**, master-mixed reagents coupled with plate-based processing for up to 576 samples in one 96-well plate
  - **For Custom Enrichment**, master-mixed reagents coupled with plate-based processing for up to 1152 samples in one 96-well plate
- Cost-effective enrichment sequencing
  - **For Exome Enrichment**, pre-enrichment pooling of up to six samples
  - **For Custom Enrichment**, pre-enrichment pooling of up to 12 samples
- Integrated solution
  - Optimized for use with TruSeq DNA Sample Preparation kits to provide convenient end-to-end enrichment sequencing solution
- Highest efficiency enrichment sequencing
  - Most comprehensive enrichment coverage, highest uniformity, and lowest DNA input requirement

The exome and custom enrichment protocols are the same, with the following exceptions called out in the procedures:

Table 1 Exome vs. Custom Enrichment

	Exome	Custom
Sample Processing	≤ 576	≤ 1152
Pre-enrichment Sample Pooling	≤ 6	≤ 12
Oligos	Capture Target (CTO)	Custom Selected (CSO)
Kit Configuration	8, 24, 48, 96, 192, 480, or 960 reactions	4, 8, 24, or 96 reactions

NOTE

To prepare libraries for enrichment, reference the Illumina *TruSeq DNA Sample Preparation Guide* for the TruSeq DNA Sample Prep kit that you are using. After completing the Enrich DNA Fragment procedures in the TruSeq DNA Sample Preparation protocol, immediately proceed to *First Hybridization* on page 32 of this guide.

What's New

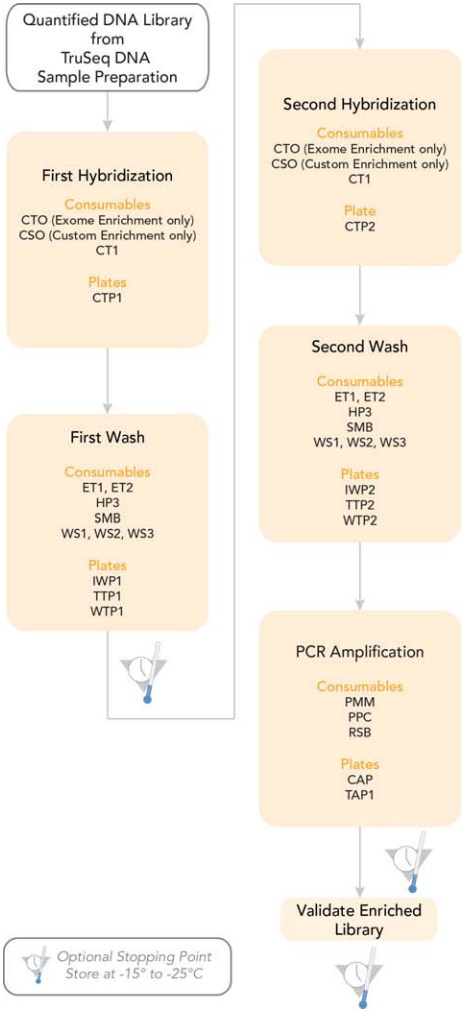
The following changes were made in this guide revision:

- Added to Best Practices:
  - Do not reuse AMPure XP beads and always add fresh beads when performing these procedures.
  - *Equipment* section
- Tracking tools:
  - Revised documentation download information
  - Added instructions for which assay to select when using the Illumina Experiment Manager
- Updated references to TruSeq DNA Sample Prep kits to include the TruSeq DNA HT Sample Preparation Kit and the name change of the *TruSeq DNA Sample Prep Kit v2* to *TruSeq DNA LT Sample Preparation Kit*.
- Added time and temperature details to hybridization procedures.
- Removed references to the use of barcode labels and replaced with instructions to write plate name on plate with a smudge resistant pen.
- Reformatted the consumables list at the start of each procedure to a table.

Enrichment Workflow

This section describes the TruSeq Enrichment workflow. The exome and custom enrichment follows the same protocol, but use different oligo reagents during the hybridization procedures.

Figure 1 TruSeq Enrichment Workflow



Introduction

Prior to TruSeq Enrichment, a human DNA library must be prepared using a TruSeq DNA Sample Prep kit.

First Hybridization

The DNA library is mixed with capture probes of targeted regions. Hybridization ensures targeted regions bind to the capture probes thoroughly. Multiple libraries can be combined with different indices into a single pool prior to enrichment.

First Wash

Streptavidin beads are used 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.

Second Hybridization

The first elution of the DNA library is mixed with capture probes of targeted regions. The second hybridization ensures the targeted regions are further enriched.

Second Wash

Streptavidin beads are used 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, similar to the First Wash.

PCR Amplification

PCR is used to amplify the enriched DNA library, or pooled DNA libraries, for sequencing and is performed with the same PCR primer cocktail used in TruSeq DNA Sample Preparation.

Enriched Library Validation

Illumina recommends performing procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Data Assessment

Following TruSeq Enrichment Illumina recommends using the TruSeqEnrichment scripts to evaluate the quality of your data. These scripts should be run after alignment with CASAVA. Reference the *TruSeq Enrichment Analysis Guide* for details.

Getting Started

This section explains standard operating procedures and precautions for performing the TruSeq Enrichment. You will also find the kit contents and lists of standard equipment and consumables.

The TruSeq Enrichment protocol described in this guide assumes that you are familiar with the contents of this section, have implemented all the recommendations, and have obtained all of the requisite equipment and consumables.

Acronyms

Table 2 TruSeq Enrichment Acronyms

Acronym	Definition
CAP	Cleaned Amplification Plate
CSO	Custom Selected Oligos
CT1	Capture Target Buffer 1
CTO	Capture Target Oligos
CTP	Capture Target Plate
dsDNA	Double-stranded DNA
ET1	Elute Target Buffer 1
ET2	Elute Target Buffer 2
HP3	2N NaOH
IWP	Intermediate Wash Plate
PCR	Polymerase Chain Reaction
PMM	PCR Master Mix, Polymerase

Acronym	Definition
PPC	PCR Primer Cocktail
PTP	Pooled Target Plate
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
TAP	Target Amplification Plate
TTP	Temporary Target Plate
WS1	Wash Solution 1
WS2	Wash Solution 2
WS3	Wash Solution 3
WTP	Wash Target Plate

Best Practices

When preparing gDNA libraries for sequencing, you should always adhere to good molecular biology practices. Read through the entire protocol prior to starting, to ensure all of the required materials are available and your equipment is programmed and ready to use.

NOTE

For more information, see the *TruSeq Sample Preparation Best Practices and Troubleshooting Guide* which you can download from the Illumina website at http://www.illumina.com. Go to the TruSeq Enrichment support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).

- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as qPCR, or those that require small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, then due diligence should be taken to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken with solutions of high molecular weight double-stranded DNA (dsDNA). These can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.
- To minimize pipetting errors, especially with small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, pipette once from the reagent tubes with a larger volume, rather than many times with small volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

Handling Magnetic Beads

Follow appropriate handling methods when working AMPure XP Beads:

NOTE
Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the Consumables and Equipment list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Prior to use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- After adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.

- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant are left when the eluates are removed from the bead pellet.
- Prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time might be required.
- Use the Resuspension Buffer (RSB) for DNA elution.
- Avoid over drying the beads, which can impact final yields.
- Resuspend the dried pellets using a single channel or multichannel pipette.
- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Target Capture

The following specifies recommended target capture methods during enrichment:

- It is advisable to make aliquots of the PCR Master Mix once it has been thawed the first time. The aliquots should be refrozen immediately for future use to reduce the potential breakdown of the components during multiple freeze-thaw cycles. This will assure consistent and reproducible results.
- The Streptavidin Magnetic Bead tubes contain metallic particles that will quickly settle to the bottom of the tube. When aliquoting Streptavidin Magnetic Bead particles, care should be taken to fully and completely mix the Streptavidin Magnetic Bead solution prior to aliquoting to assure an equal distribution of particles across samples.

- The components of the Capture Target Buffer 1 tubes stored at -15° to -25°C will be cloudy upon thawing. It is important to make sure the Capture Target Buffer 1 solution, once equilibrated to room temperature, is thoroughly vortexed and visually inspected for remnant cloudiness and crystals prior to use. If the Capture Target Buffer 1 solution is not clear it should be vortexed until the solution is completely clear.
- Care should be taken during all mixing steps to avoid the creation of foam in the solutions, as the formation of bubbles might interfere with optimal biochemical conditions and result in significantly reduced yields. Set the pipette to a volume just below the final volume to be mixed to avoid aspirating air, which can introduce bubbles into the mixture.
- Due to the high-throughput, multi-well character of the protocol, it is very important to avoid cross-contamination from well to well. Plates should not be vortexed but rather mixed with multichannel pipettes. Plates and samples should be centrifuged if solutions have adhered to the tube walls. For small sample numbers it is advisable to leave an empty well between samples, which significantly reduces the potential for cross contamination.
- When applying a Microseal 'B' adhesive seal, care should be taken to assure a very tight seal over all wells and to avoid folds in the seal. The use of an adhesive seal roller is recommended as well as a visual examination of complete attachment around each well. The Microseal 'B' adhesive seal was chosen for its exceptionally strong adherence. Due to this characteristic, it is highly recommended to secure a plate on a 96-well plate tray prior to removal of the seal, as the removal of the seal might cause the plate to be inadvertently dropped or shaken. This can lead to loss of samples or cross contamination.
- Certain steps of the protocol will result in volumes that are larger than 100 µl (e.g.,200 µl). Incubations are recommended to occur on thermal cyclers that might not be programmable for volumes larger than 100 µl. This is not a problem for the enrichment process and should not be a cause of concern.

Equipment

Review the programming instructions for your thermal cycler user guide to ensure that it is programmed appropriately using the heated lid function.

DNA Input Recommendations

It is important to quantitate the DNA library from your TruSeq DNA sample preparation and assess the DNA quality prior to performing TruSeq Enrichment.

Input DNA Library Quality

The DNA library quality is important for the success of the TruSeq Enrichment assay. It is highly recommended to verify the size distribution of the input library prior to enrichment by running an aliquot on a gel or an Agilent Technologies 2100 Bioanalyzer. When running samples on an Agilent High Sensitivity DNA chip, load 1µl of a 1:50 dilution of your library. The following are example traces of a final DNA library made through either the gel or gel-free method when run on a High Sensitivity DNA chip.

Figure 2 Gel Library on High Sensitivity DNA Chip

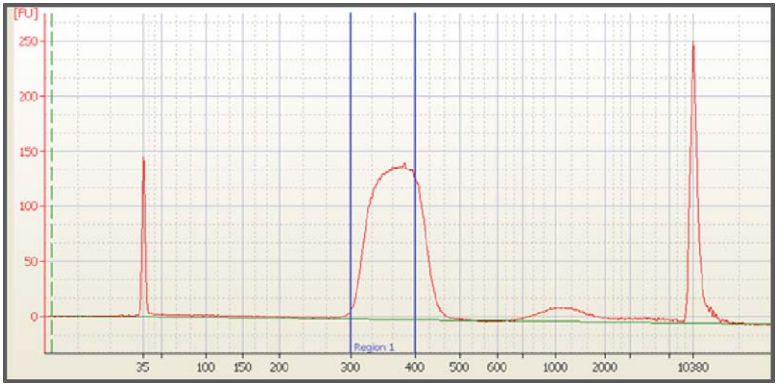
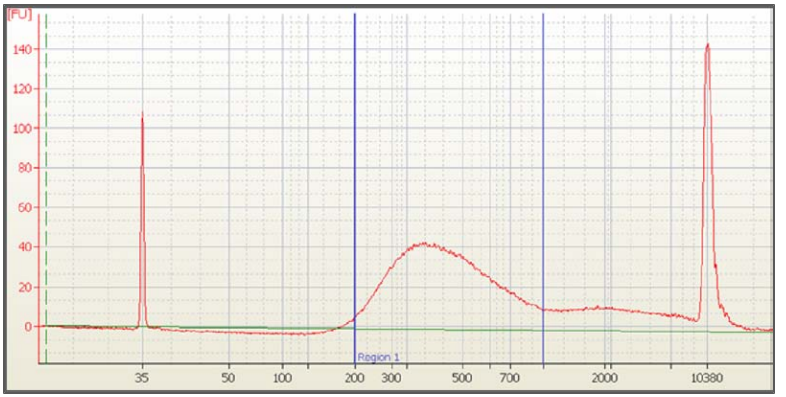


Figure 3 Gel-free Library on High Sensitivity DNA Chip



Input DNA Library Quantitation

Illumina recommends 500 ng per library for the TruSeq Enrichment protocol. 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.

To obtain an accurate quantification of the DNA library, it is recommended to quantify the starting DNA library using a fluorometric based method such as the Qubit dsDNA BR Assay system. Illumina recommends using 2 µl of each DNA sample with 198 µl of the Qubit working solution for sample quantification.

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 TruSeq Enrichment documents from the Illumina website at http://www.illumina.com. Go to the TruSeq Enrichment support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

- **Illumina Experiment Manager (IEM)** 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, dual indices, and other parameters applicable to your 96-well plate.
  - When prompted to select an Assay in IEM, choose:
    - **TruSeq LT** if you are using a TruSeq DNA LT Sample Prep Kit
    - **TruSeq HT** if you are using the TruSeq DNA HT Sample Prep Kit

NOTE
IEM can be run on any Windows platform. You can download it from the Illumina website at http://www.illumina.com. A MyIllumina account is required.

Kit Contents

The TruSeq Exome Enrichment and TruSeq Custom Enrichment protocols each require a separate kit. Check to make sure that you have all of the reagents identified in this section for the protocol that you are performing before proceeding to the enrichment procedures.

Table 3 TruSeq Enrichment Kit Reaction Configurations

Number of Reactions Supported	Exome	Custom
x4		X
x8	X	X

Number of Reactions Supported	Exome	Custom
x24	X	X
x48	X	
x96	X	X
x192	X	
x480	X	
x960	X	

NOTE
For larger size studies, TruSeq Custom Enrichment Kits can be ordered in multiples of 24 and 96 reactions.

TruSeq Exome Enrichment Kit

The TruSeq Exome Enrichment kits are available in the following configurations. Each kit contains two boxes of reagents.

Table 4 TruSeq Exome Enrichment Kits

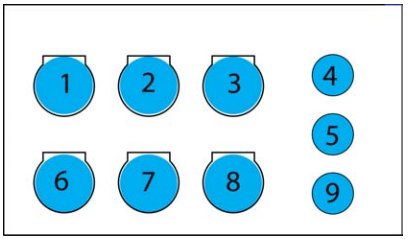
Catalog #	Number of Reactions Supported	Number of Samples Supported	Box Configuration
FC-121-1008	8	48	1 x 8 reaction boxes
FC-121-1024	24	144	3 x 8 reaction boxes
FC-121-1048	48	288	6 x 8 reaction boxes
FC-121-1096	96	576	1 x 96 reaction boxes
FC-121-1192	192	1,152	2 x 96 reaction boxes
FC-121-1480	480	2,880	5 x 96 reaction boxes
FC-121-1960	960	5,760	10 x 96 reaction boxes

8-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 4 TruSeq Exome Enrichment Kit, 8 (Box 1 of 2), part # 15017727



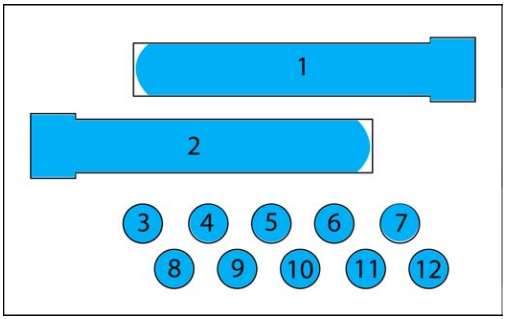
Slot	Reagent	Part #	Description
1–2	TC#-SMB	15015927	Streptavidin Magnetic Beads
3	TC#-WS1	15015775	Wash Solution 1
4	TC#-RSB	15018075	Resuspension Buffer
5	TC#-ET2	15013008	Elute Target Buffer 2
6	TC#-WS1	15015775	Wash Solution 1
7–8	TC#-WS3	15015933	Wash Solution 3
9	TC#-ET2	15013008	Elute Target Buffer 2

8-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 5 TruSeq Exome Enrichment Kit, 8 (Box 2 of 2), part # 15017728



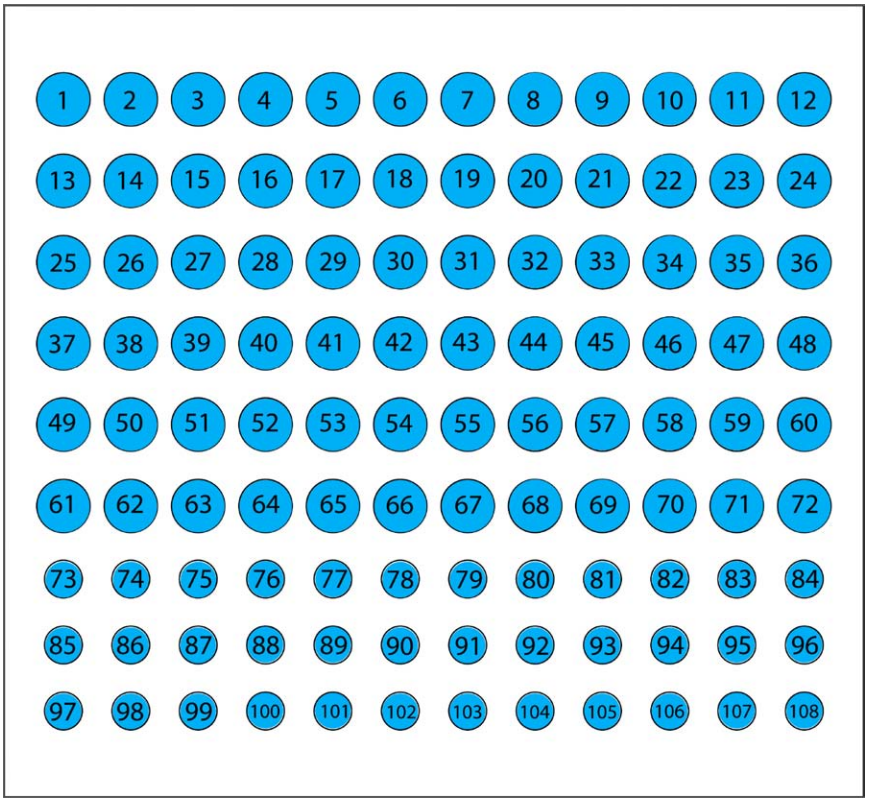
Slot	Reagent	Part #	Description
1–2	TC#-WS2	15015898	Wash Solution 2
3–4	TC#-CT1	15015770	Capture Target Buffer 1
5–6	TC#-ET1	15013006	Elute Target Buffer 1
7–8	TC#-CTO	15013000	Capture Target Oligos
9–10	TC#-HP3	11324596	2N NaOH
11	TC#-PMM	15017038	PCR Master Mix, Polymerase
12	TC#-PPC	15021793	PCR Primer Cocktail

96-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 6 TruSeq Exome Enrichment Kit, 96 (Box 1 of 2), part # 15019321



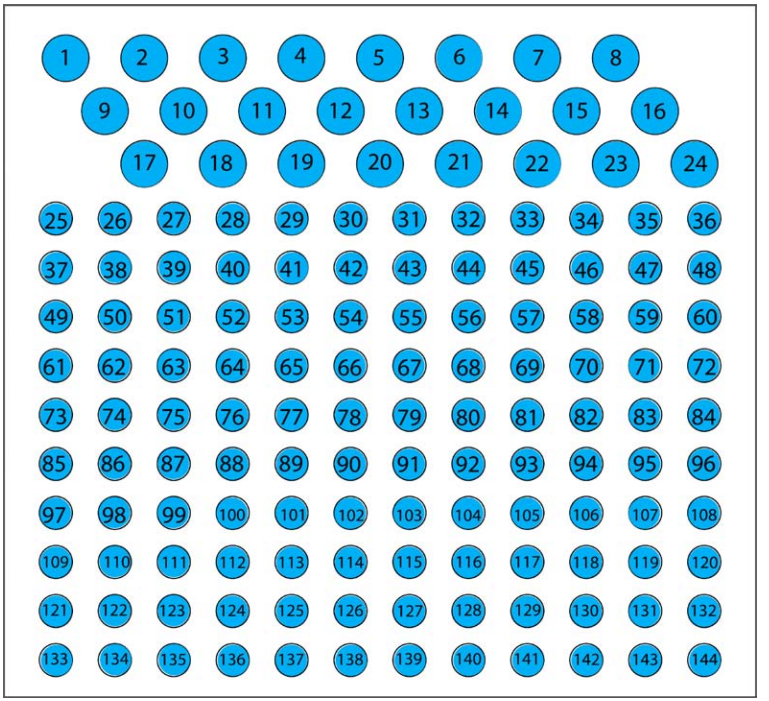
Slot	Reagent	Part #	Description
1–24	TC#-SMB	15015927	Streptavidin Magnetic Beads
25–48	TC#-WS1	15015775	Wash Solution 1
49–72	TC#-WS3	15015933	Wash Solution 3
73–96	TC#-ET2	15013008	Elute Target Buffer 2
97–108	TC#-RSB	15018075	Resuspension Buffer

96-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 7 TruSeq Exome Enrichment Kit, 96 (Box 2 of 2), part # 15019322



Slot	Reagent	Part #	Description
1–24	TC#-WS2	15015898	Wash Solution 2
25–48	TC#-CT1	15015770	Capture Target Buffer 1
49–72	TC#-ET1	15013006	Elute Target Buffer 1
73–96	TC#-CTO	15013000	Capture Target Oligos
97–120	TC#-HP3	11324596	2N NaOH
121–132	TC#-PMM	15017038	PCR Master Mix, Polymerase
133–144	TC#-PPC	15021793	PCR Primer Cocktail

TruSeq Custom Enrichment Kit

The TruSeq Custom Enrichment kits are available in the following configurations. Each kit contains two boxes of reagents and a box of custom oligos.

Table 5 TruSeq Custom Enrichment Kits

Catalog #	Number of Reactions Supported	Number of Samples Supported
FC-123-1004	4	48
FC-123-1008	8	96
FC-123-1024	24	288
FC-123-1096	96	1,152

NOTE
For larger size studies, TruSeq Custom Enrichment Kits can be ordered in multiples of 24 and 96 reactions.

Custom Oligos Box

All TruSeq Custom Enrichment kits contain a box of custom oligos.

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store this box at -15° to -25°C.

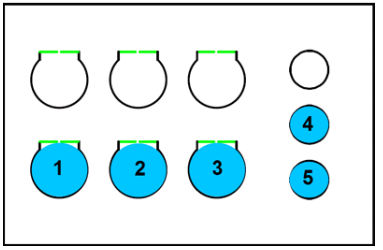
This box, part # 15022752, contains Custom Target Oligos (TC#-CSO), part # 15022753 and a lot number specific to each customer order. The boxes are configured for 6, 24, 120, and 240 slots. The number of occupied slots is dependent upon the pulldowns that were ordered with your custom kit.

4-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 8 TruSeq Custom Enrichment Kit, 4 Rxn (Box 1 of 2), part # 15032360



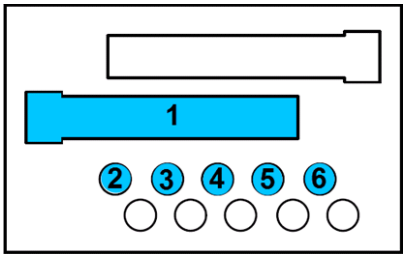
Slot	Reagent	Part #	Description
1	TC#-SMB	15015927	Streptavidin Magnetic Beads
2	TC#-WS1	15015775	Wash Solution 1
3	TC#-WS3	15015933	Wash Solution 3
4	TC#-ET2	15013008	Elute Target Buffer 2
5	TC#-RSB	15018075	Resuspension Buffer

4-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 9 TruSeq Custom Enrichment Kit, 4 Rxn (Box 2 of 2), part # 15032361



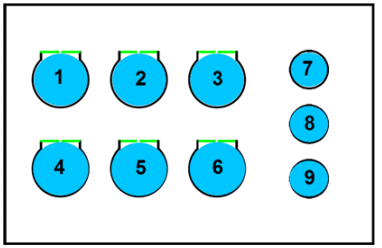
Slot	Reagent	Part #	Description
1	TC#-WS2	15015898	Wash Solution 2
2	TC#-CT1	15015770	Capture Target Buffer 1
3	TC#-ET1	15013006	Elute Target Buffer 1
4	TC#-HP3	11324596	2N NaOH
5	TC#-PPC	15031752	PCR Primer Cocktail
6	TC#-PMM	15017038	PCR Master Mix, Polymerase

8-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 10 TruSeq Custom Enrichment Kit, 8 Rxn (Box 1 of 2), part # 15032357



Slot	Reagent	Part #	Description
1–2	TC#-SMB	15015927	Streptavidin Magnetic Beads

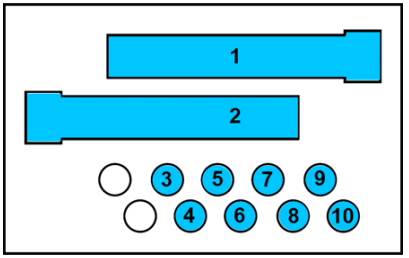
Slot	Reagent	Part #	Description
3–4	TC#-WS1	15015775	Wash Solution 1
5–6	TC#-WS3	15015933	Wash Solution 3
7–8	TC#-ET2	15013008	Elute Target Buffer 2
9	TC#-RSB	15018075	Resuspension Buffer

8-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 11 TruSeq Custom Enrichment Kit, 8 Rxn (Box 2 of 2), part # 15032358



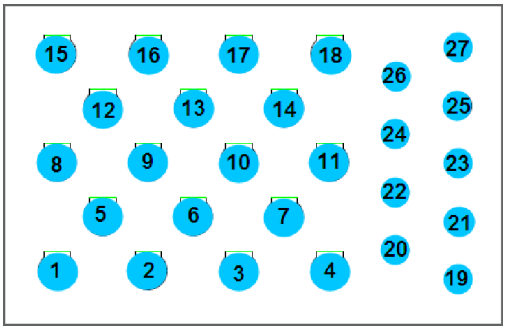
Slot	Reagent	Part #	Description
1–2	TC#-WS2	15015898	Wash Solution 2
3–4	TC#-CT1	15015770	Capture Target Buffer 1
5–6	TC#-ET1	15013006	Elute Target Buffer 1
7–8	TC#-HP3	11324596	2N NaOH
9	TC#-PPC	15031752	PCR Primer Cocktail
10	TC#-PMM	15017038	PCR Master Mix, Polymerase

24-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 12 TruSeq Custom Enrichment Kit, 24, Box 1 of 2, part # 15022030



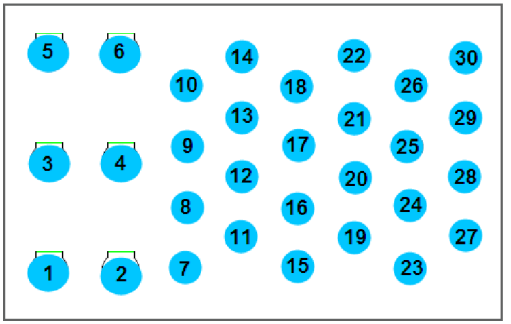
Slot	Reagent	Part #	Description
1–6	TC#-SMB	15015927	Streptavidin Magnetic Beads
7–12	TC#-WS1	15015775	Wash Solution 1
13–18	TC#-WS3	15015933	Wash Solution 3
19–24	TC#-ET2	15013008	Elute Target Buffer 2
25–27	TC#-RSB	15018075	Resuspension Buffer

24-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 13 TruSeq Custom Enrichment Kit, 24, Box 2 of 2, part # 15022031



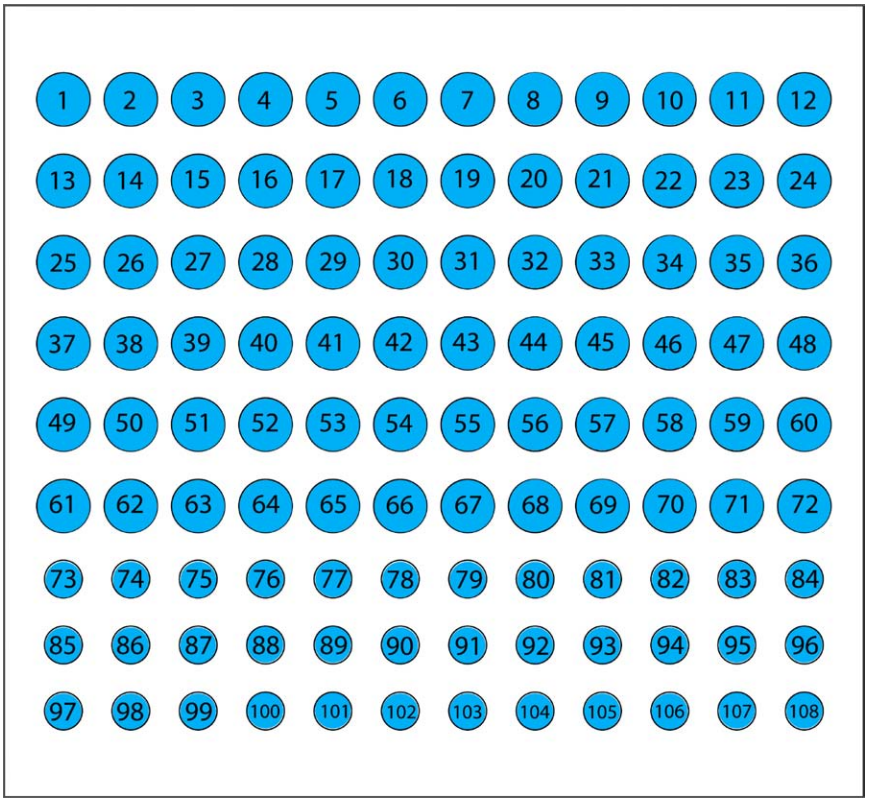
Slot	Reagent	Part #	Description
1–6	TC#-WS2	15015898	Wash Solution 2
7–12	TC#-CT1	15015770	Capture Target Buffer 1
13–18	TC#-ET1	15013006	Elute Target Buffer 1
19–24	TC#-HP3	11324596	2N NaOH
25–27	TC#-PMM	15017038	PCR Master Mix, Polymerase
28–30	TC#-PPC	15021793	PCR Primer Cocktail

96-Reaction, Box 1

Store at 2° to 8°C

This box is shipped at room temperature. As soon as you receive your kit, store the following components at 2° to 8°C.

Figure 14 TruSeq Custom Enrichment Kit, 96, Box 1 of 2, part # 15022163



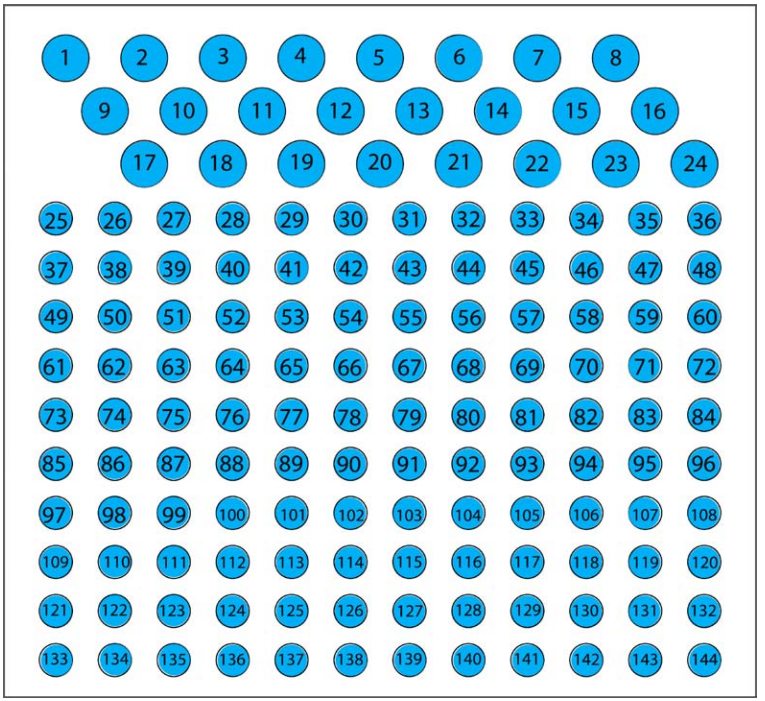
Slot	Reagent	Part #	Description
1–24	TC#-SMB	15015927	Streptavidin Magnetic Beads
25–48	TC#-WS1	15015775	Wash Solution 1
49–72	TC#-WS3	15015933	Wash Solution 3
73–96	TC#-ET2	15013008	Elute Target Buffer 2
97–108	TC#-RSB	15018075	Resuspension Buffer

96-Reaction, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.

Figure 15 TruSeq Custom Enrichment Kit, 96, Box 2 of 2, part # 15022169



Slot	Reagent	Part #	Description
1–24	TC#-WS2	15015898	Wash Solution 2
25–49	TC#-CT1	15015770	Capture Target Buffer 1
50–74	TC#-ET1	15013006	Elute Target Buffer 1
75–99	TC#-HP3	11324596	2N NaOH
100–112	TC#-PMM	15017038	PCR Master Mix, Polymerase
113–125	TC#-PPC	15021793	PCR Primer Cocktail
126–144	Empty		

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to enrichment.

Table 6 User-Supplied Consumables

Consumable	Supplier
10 µl multichannel pipettes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl single channel pipettes	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl single channel pipettes	General lab supplier
300 µl 96-well skirtless PCR plates or Twin.Tec 96-well PCR plates	E&K Scientific, part # 480096 or Eppendorf, part # 951020303
96-well storage plates, round well, 0.8m (MIDI plates)	Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Agencourt AMPure XP, 60 ml kit	Beckman Coulter Genomics, part # A63881/A63880
Ethanol 200 proof (absolute) for molecular biology	Sigma Aldrich, part # E7023
Microseal 'B' adhesive seals	Bio-Rad, part # MSB1001

Consumable	Supplier
PCR grade water	General lab supplier
PCR tubes	General lab supplier
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

Table 7 User-Supplied Equipment

Equipment	Supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[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
Magnetic stand-96	Ambion, part # AM10027
Microcentrifuge	General lab supplier
Plate 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

TruSeq DNA Sample Prep

Prior to TruSeq Enrichment, you must first prepare a human DNA library using a TruSeq DNA Sample Prep kit (v1, LT, or HT) (see the Illumina *TruSeq DNA Sample Preparation Guide* for the TruSeq DNA Sample Prep kit that you are using). TruSeq DNA sample prep provides an option for gel-free vs. with gel to support enrichment. After completing the Enrich DNA Fragment procedures in the TruSeq DNA Sample Preparation protocol, immediately proceed to *First Hybridization* on page 32. Review *Best Practices* on page 9 before proceeding.

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
Capture Target Buffer 1 (CT1)	1 tube	-15° to -25°C	Illumina
For Custom Enrichment: Custom Selected Oligos (CSO)	1 tube	-15° to -25°C	Illumina
For Exome Enrichment: Capture Target Oligos (CTO)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	1 plate		User
DNA library output from Illumina TruSeq DNA Sample Prep Kit	500 ng per DNA library		User
Microseal 'B' Adhesive Seal	1 seal		User
PCR Grade Water	As needed		User

Preparation

- Do one of the following:
  - For Custom Enrichment, remove the Custom Selected Oligos tube from -15° to -25°C storage and thaw on ice.
  - For Exome Enrichment, remove the Capture Target Oligos tube from -15° to -25°C storage and thaw on ice.

- Remove the Capture Target Buffer 1 tube from -15° to -25°C storage and thaw at room temperature.
- Pre-program the thermal cycler as follows:
  a Choose the pre-heat lid option and set to 100°C
  b 95°C for 10 minutes
  c 18 cycles of 1 minute incubations, starting at 93°C, then decreasing 2°C per cycle
  d 58°C for forever
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **CTP1** with a smudge resistant pen.

Make PTP and Resuspend PTP

1 Reference the table below for the amount of DNA libraries to use for enrichment. Illumina recommends using 500 ng of each DNA library, quantified by the Qubit Fluorometric Quantitation system. See *Input DNA Library Quantitation* on page 14. 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.

Table 8 DNA Libraries for Enrichment

Exome	Custom	Library Pool Complexity	Total DNA Library Mass (ng)
X	X	1-plex	500
X	X	2-plex	1000
X	X	3-plex	1500
X	X	4-plex	2000
X	X	5-plex	2500
X	X	6-plex	3000
X	X	7-plex	3500

Exome	Custom	Library Pool Complexity	Total DNA Library Mass (ng)
X	X	8-plex	4000
X	X	9-plex	4500
X	X	10-plex	5000
X	X	11-plex	5500
X	X	12-plex	6000

Make CTP1

1 Vortex the Capture Target Buffer 1 tube for 5 seconds. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the Capture Target Buffer 1 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 10–20 times to mix thoroughly. Multiply each volume by the number of sample pools being prepared. Prepare 5% extra reagent mix if you are preparing multiple pooled samples.

Reagent	Volume (µl)
Diluted DNA library	40
Capture Target Buffer 1	50
For Custom Enrichment use Custom Selected Oligos For Exome Enrichment use Capture Target Oligos	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 to 280 xg for 1 minute.

2 Place the sealed CTP1 plate on the pre-programmed thermal cycler. Close the lid and incubate using the pre-programmed settings:
   a Pre-heated lid set to 100°C
   b 95°C for 10 minutes
   c 18 cycles of 1 minute incubations, starting at 93°C, then 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
2N NaOH (HP3)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 1 (ET1)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2° to 8°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2° to 8°C	Illumina
Wash Solution 1 (WS1)	1 tube	2° to 8°C	Illumina
Wash Solution 2 (WS2)	1 tube	-15° to -25°C	Illumina
Wash Solution 3 (WS3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate	1 plate		User
300 µl 96-well skirtless PCR Plates or twin.tech 96-well PCR Plates	2 plates		User
Microseal 'B' Adhesive Seals	5 seals		User
PCR Grade Water	As needed		User
PCR Tubes	1 per sample		User

Preparation

- Remove the Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes from 2° to 8°C storage and let stand at room temperature.
- Remove the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 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** with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **TTP1** with a smudge resistant pen.
- Label a new 96-well MIDI plate **WTP1** with a smudge resistant pen.
- [Optional] Label one new PCR tube per sample "First Elution for qPCR" with a smudge resistant pen.

Make WTP1

1 Remove the CTP1 plate from the thermal cycler.

2 Centrifuge the CTP1 plate to 280 xg 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 Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl of well-mixed Streptavidin Magnetic Beads to the wells of the WTP1 plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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 to 280 xg 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 Vortex the Wash Solution 1 tube for 5 seconds. Visually make sure that no crystal structures are present.

NOTE
If crystals are observed, vortex the Wash Solution 1 tube until no crystal structures are visible.

2 Add 200 µl Wash Solution 1 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 Vortex the Wash Solution 2 tube for 5 seconds. Visually make sure that the Wash Solution 2 is mixed thoroughly.

2 Add 200 µl Wash Solution 2 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 Wash Solution 2 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 Wash Solution 2 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 Wash Solution 3 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 Wash Solution 3, 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 Wash Solution 3.

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 sample pools being prepared. Prepare 5% extra reagent mix if you are preparing multiple sample pools.

Reagent	Volume (µl)
Elute Target Buffer 1	28.5
2N NaOH	1.5
Total Volume per Sample	**30**

2 Remove the IWP1 plate from the magnetic stand.

3 Add 30 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 to 280 xg 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 29 µl of supernatant from each well of the IWP1 plate to the corresponding well of the new 96-well PCR plate labeled TTP1.

10 Add 5 µl Elute Target Buffer 2 to each well of the TTP1 plate containing samples to neutralize the elution. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.

11 Seal the TTP1 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 [Optional] Dilute 2 µl of the first elution in 98 µl PCR grade water (1:50 dilution) in a new PCR tube labeled "First Elution for qPCR". Cap each tube and store at -15° to -25°C.

NOTE
The First Elution for qPCR tube is used for yield quantification.

13 Store the remaining reagents as follows:
   a Place the Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes in 2° to 8°C storage.
   b Place the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 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 43 immediately, the protocol can be safely stopped here. If you are stopping, seal the TTP1 plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days. When proceeding, thaw the TTP1 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
Capture Target Buffer 1 (CT1)	1 tube	-15° to -25°C	Illumina
For Custom Enrichment: Custom Selected Oligos (CSO)	1 tube	-15° to -25°C	Illumina
For Exome Enrichment: Capture Target Oligos (CTO)	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	1 seal		User
PCR Grade Water	As needed		User

Preparation

- Do one of the following:
  - For Exome Enrichment, remove the Capture Target Oligos tube from -15° to -25°C storage and thaw on ice.
  - For Custom Enrichment, remove the Custom Selected Oligos tube from -15° to -25°C storage and thaw on ice.
- Remove the Capture Target Buffer 1 tube from -15° to -25°C storage and thaw at room temperature.
- Remove the TTP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *First Wash* on page 36 and let stand to thaw at room temperature.

- Centrifuge the thawed TTP1 plate to 280 xg for 1 minute
- Remove the adhesive seal from the thawed TTP1 plate.

▸ Pre-program the thermal cycler as follows:

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

b 95°C for 10 minutes

c 18 cycles of 1 minute incubations, starting at 93°C, then decreasing 2°C per cycle

d 58°C for forever

▸ Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **CTP2** with a smudge resistant pen.

Add CTO/CSO

1 Vortex the Capture Target Buffer 1 tube for 5 seconds. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the Capture Target Buffer 1 tube until it appears clear.

2 In the order listed below, add the following to each well of the new 300 µl 96-well PCR plate labeled CTP2. Gently pipette the entire volume up and down 10-20 times to mix thoroughly. Multiply each volume by the number of samples being prepared. Prepare 5% extra reagent mix if you are preparing multiple samples. Change the tip after each sample.

Reagent	Volume (µl)
Capture Target Buffer 1	50
For Custom Enrichment use Custom Selected Oligos For Exome Enrichment use Capture Target Oligos	10
PCR Grade Water	10
First Elution from TTP1 Plate	30
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 to 280 xg for 1 minute.
2. Place the sealed CTP2 plate on the pre-programmed thermal cycler. Close the lid and incubate using the pre-programmed settings:
   a. Pre-heated lid set to 100°C
   b. 95°C for 10 minutes
   c. 18 cycles of 1 minute incubations, starting at 93°C, then 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 36.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 1 (ET1)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2° to 8°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2° to 8°C	Illumina
Wash Solution 1 (WS1)	1 tube	2° to 8°C	Illumina
Wash Solution 2 (WS2)	1 tube	-15° to -25°C	Illumina
Wash Solution 3 (WS3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate	1 plate		User
300 µl 96-well skirtless PCR Plates or twin.tech 96-well PCR Plates	2 plates		User
Microseal 'B' Adhesive Seals	5 seals		User
PCR Grade Water	As needed		User
PCR Tubes	1 per sample		User

Preparation

- Remove the Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes from 2° to 8°C storage and let stand at room temperature.
- Remove the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 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** with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **TTP2** with a smudge resistant pen.
- Label a new 96-well MIDI plate **WTP2** with a smudge resistant pen.
- [Optional] Label one new PCR tube per sample "Second Elution for qPCR" with a smudge resistant pen.

Make WTP2

1 Remove the CTP2 plate from the thermal cycler.

2 Centrifuge the room temperature CTP2 plate to 280 xg 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 Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl of well-mixed Streptavidin Magnetic Beads 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 to 280 xg 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 Vortex the Wash Solution 1 tube for 5 seconds. Visually make sure that no crystal structures are present.

NOTE
If crystals are observed, vortex the Wash Solution 1 tube until no crystal structures are visible.

2 Add 200 µl Wash Solution 1 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 Vortex the Wash Solution 2 tube for 5 seconds. Visually make sure that the Wash Solution 2 is mixed thoroughly.

2 Add 200 µl Wash Solution 2 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 Wash Solution 2 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 Wash Solution 2 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 Wash Solution 3 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 Wash Solution 3, 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 Wash Solution 3.

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 sample pools being prepared. Prepare 10% extra reagent mix if you are preparing multiple sample pools.

Reagent	Volume (µl)
Elute Target Buffer 1	28.5
2N NaOH	1.5
Total Volume per Sample	**30**

2 Remove the IWP2 plate from magnetic stand

3 Add 30 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 to 280 xg 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 29 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 5 µl Elute Target Buffer 2 to each well of the TTP2 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 TTP2 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 Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes in 2° to 8°C storage.
   b Place the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 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. PCR is performed with the same PCR primer cocktail used in TruSeq DNA Sample Preparation.

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube	-15° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
300 µl 96-well skirtless PCR Plates or twin.tech 96-well PCR Plates	2 plates		User
AMPure XP Beads	1 bottle		User
Freshly Prepared 80% Ethanol (EtOH)	As needed		User
Microseal 'B' Adhesive Seals	3 seals		User

Preparation

- Remove one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage to thaw, then place the tubes on ice.

  NOTE
  If you do not intend to consume the PCR Master Mix and PCC 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 PCR Primer Cocktail and PCR Master Mix tubes for 5 seconds.
- Review *Handling Magnetic Beads* on page 10.

- 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:
  a Choose the pre-heat lid option and set to 100°C
  b 98°C for 30 seconds
  c 10 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
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **CAP** with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate **TAP1** with a smudge resistant pen.

Add PPC

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

Reagent	Volume (µl)
Second Elution from TTP2 plate	20
PCR Master Mix	25
PCR Primer Cocktail	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 to 280 xg for 1 minute.

Amp PCR

1 Place the sealed TAP1 plate on the pre-programmed thermal cycler, close the lid, and incubate the plate.

Make CAP

NOTE
Before performing clean up, *Handling Magnetic Beads* on page 10 when working with AMPure XP Beads.

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 Resuspension Buffer. 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 30 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 56 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*.

[Optional] To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the enriched library on a gel.

Controls

This section describes the sample-independent and sample-dependent controls used in TruSeq Enrichment.

Sample-Independent Controls

During the sample preparation process, artificial dsDNA targets (CTE, CTA, CTL) are incorporated to act as controls for the enzymatic activities of the ERP, ATL and LIG reagents. Identification of these controls by sequencing is indicative of the success of a particular enzymatic step in the library preparation process (Reference the *In-Line Control DNA* Appendix of your *TruSeq DNA Sample Preparation Guide*. To enrich for these sample prep controls, Illumina has included a set of probes in the oligo pool to target these dsDNA controls. The control reagents can be used for a variety of library insert sizes. Each is provided in ladders ranging from approximately 150-850 bp in 100 bp increments. Each targeted control molecule has a unique DNA sequence indicating both its function and size. The RTA software (version 1.9 and higher) recognizes these sequences, isolates the control sequences from the main body of sequencing reads and reports their counts per lane in the controls tab of the RTA status.html page.

From a starting library input of 500 ng that underwent the enrichment process, you can expect approximately 10–100 control reads per tile for each of the four control types as indicative of a successful library preparation process and subsequent enrichment. The absence of reads for an individual control type is indicative of failure at that particular step of the library preparation process. However, the absence of reads for all four control types likely indicates a failure during the enrichment process particularly if the library was validated prior to starting the enrichment process.

Sample-Dependent Controls

A panel of probes has been included in the CTO and CSO probe pool, which functions as sample dependent controls for the enrichment process.

GC Control Probes

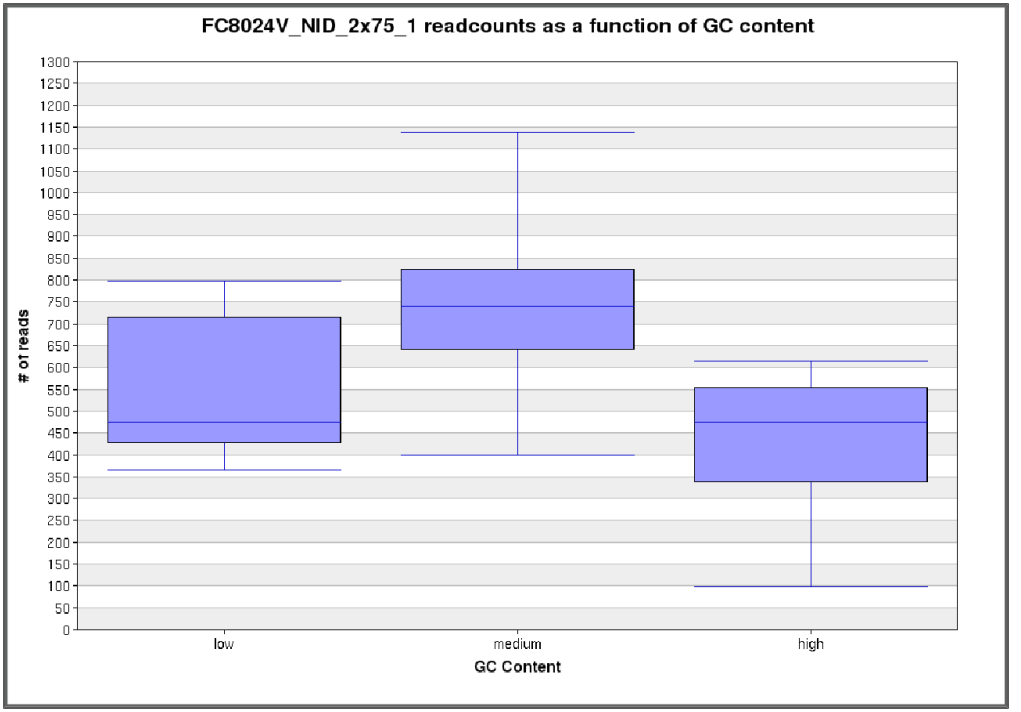
A subset of these probes target non-polymorphic regions of the human genome and function to assess assay stringency across GC content. The performance of these

stringency controls is depicted in the analysis output file in which the readcounts for each probe is plotted across %GC (see sample output file). Ideally, the read counts among the low (29–33% GC), medium (41-50% GC), and high (60–72% GC) classes should be similar. As a result, changes affecting assay stringency will result in changes in the distribution of these control probes which likely reflects similar changes in the performance of the enrichment probe pool. For example, an increase in stringency of the assay (e.g., a greater than 42°C WS2 wash) can result in a decrease in performance (i.e., lower number of reads) of the low GC control probes.

The *<TruSeqEnrichmentRunId>_gc_controls_readcounts.png* displays a set of GC control probes. These probes were selected to have low, medium, or high GC content and the regions are independent of the probes targeting the enrichment.

The plot shows the total number of reads in the gc control regions. Each box shows the 25-75th percentile of the data and the whiskers show the minimum and maximum values. The low, medium, and high stringency boxes should be roughly similar to each other. The raw data used to generate this plot is in the *<TruSeqEnrichmentRunId>_gc_controls_readcounts.txt* file.

Figure 16 GC Control Probes Plot



Each read that overlaps by at least one base with the probe region is counted. This data is derived directly from the export.txt files. Duplicates are not removed. For each stringency level, the readcounts for every probe at that stringency level is used to generate the box and whiskers. The box and whisker is generated based on min, 25th percentile, median, 75th percentile, and max.

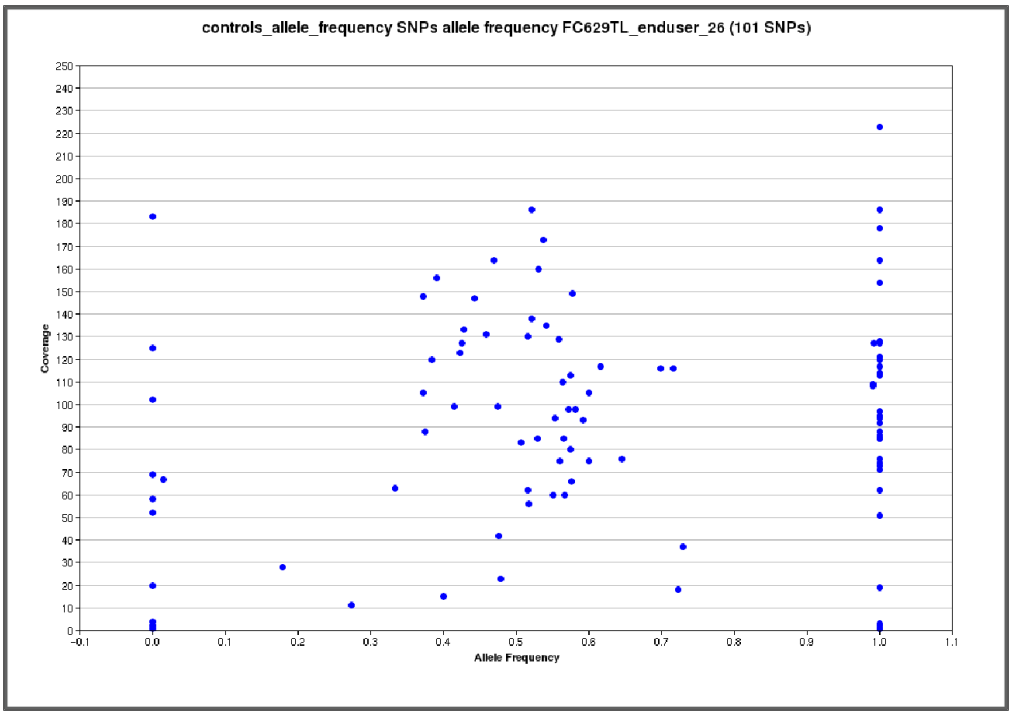
SNP Control Probes

Another subset of the sample-dependent control probes assesses for the ability to enrich for multiple alleles equally. This control panel consists of 90 probes targeting known SNPs of high minor allele frequency that are spaced far apart. The performance of these control probes is depicted in the Allele Frequency Plot which is generated as one of the TruSeq script output files. Plotted as allele frequency vs. coverage, ideally the distribution of these 90 SNPs should result in three distinct populations related to either a homozygous or heterozygous SNP. Conditions

resulting in allelic bias will result in a more widespread distribution of the 90 control SNPs and can reflect the ability to accurately call a SNP following enrichment.

The *<TruSeqEnrichmentRunId>_controls_allele_frequency.png* file displays a set of control probes targeted at known SNPs that are not in any targeted region. These probes are designed to assess the allelic bias of the assay. On genomic DNA from a homogeneous population of cells, the true allele frequency for all these SNPs is expected to be 0, 0.5, or 1.

Figure 17 SNP Control Probes



This plot shows the actual allele frequency calculated by CASAVA for each of the control probes. The X axis is the allele frequency and the y axis is the coverage. At lower coverage levels, it is expected to see a larger spread of values around the 0.5 allele frequency.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 9 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 10 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 http://www.illumina.com/msds.

Product Documentation

You can obtain PDFs of additional product documentation from the Illumina website. Go to http://www.illumina.com/support and select a product. To download documentation, you will be asked to log in to MyIllumina. After you log in, you can view or save the PDF. To register for a MyIllumina account, please visit https://my.illumina.com/Account/Register.

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