illumina®

TruSeq™ Enrichment Guide

TruSeq™

FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY
Catalog # FC-930-1012
Part # 15013230 Rev. E
June 2011

Notice

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and

are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY.

ILLUMINA DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN (INCLUDING PARTS THEREOF OR SOFTWARE) OR ANY USE OF SUCH PRODUCT(S) OUTSIDE THE SCOPE OF THE EXPRESS WRITTEN LICENSES OR PERMISSIONS GRANTED BY ILLUMINA IN CONNECTION WITH CUSTOMER'S ACQUISITION OF SUCH PRODUCT(S).

FOR RESEARCH USE ONLY

© 2010–2011 Illumina, Inc. All rights reserved.

Illumina, illumina*Dx*, BeadArray, BeadXpress, cBot, CSPro, DASL, Eco, Genetic Energy, GAIIx, Genome Analyzer, GenomeStudio, GoldenGate, HiScan, HiSeq, Infinium, iSelect, MiSeq, Nextera, Sentrix, Solexa, TruSeq, VeraCode, the pumpkin orange color, and **the Genetic Energy streaming bases** are registered trademarks or trademarks of Illumina, Inc. All other brands and names contained herein are the property of their respective owners.

Revision History

Part #	Revision	Date	Description of Change
15013230	E	June 2011	• Incorporated custom enrichment kit and protocol information. • Included 96-sample exome kit box contents and layout. • Added BioAnalyzer trace examples to Input DNA Library Quantitation. • Added plate barcode labeling instructions and referenced plates by name in procedures. • Modified procedure subheadings to correspond with plate names. • Removed wording specifying use of single channel or multichannel pipette. • Update Assessment and Controls content to support both exome and custom enrichment. • Sample-Independent Controls now supported by RTA software version 1.9 and higher. • Updated customer support.
15013230	D	March 2011	• Incorporate gel-free method • Increase Second Elution reagent volume to 20 µl for PCR Amplification.

Part #	Revision	Date	Description of Change
15013230	C	December 2010	• DNA Input Recommendation input library dilution changed to 1:50 • First Wash, WS3 Cleanup, final step - modified text to "Remove and discard any residual supernatant..." • Amp PCR, first step - Removed instruction to close thermal cycler lid when amplifying the PCR plate. • Clean Up PCR, step 9 - Resuspend the dried pellet in the each well with 30 µl Resuspension Buffer using a single channel or multichannel pipette. (added "single channel") • Script changes
15013230	B	November 2010	Specify DNA input quantitation using Agilent Bioanalyzer instead of qPCR
15013230	A	November 2010	Initial release of TruSeq Exome Enrichment protocol

Table of Contents

Notice . . . iii

Revision History . . . v

Table of Contents . . . vii

List of Tables . . . ix

Chapter 1 Overview . . . 1

Introduction . . . 2
Enrichment Workflow . . . 4

Chapter 2 Getting Started . . . 7

Introduction . . . 8
Acronyms . . . 9
Best Practices . . . 10
DNA Input Recommendations . . . 14
Tracking Tools . . . 16
Kit Contents . . . 18
Consumables and Equipment . . . 28

Chapter 3 Enrichment Protocol . . . 31

Introduction . . . 32
TruSeq DNA Sample Prep . . . 33
First Hybridization . . . 34
First Wash . . . 37
Second Hybridization . . . 43
Second Wash . . . 45

PCR Amplification 51
Enriched Library Validation 55

Appendix A Data Assessment 57

Introduction 58
Script Installation 59
Script Usage 60
Statistics 64
Coverage 70

Appendix B Controls 71

Introduction 72
Sample-Independent Controls 73
Sample-Dependent Controls 74

Technical Assistance 79

List of Tables

Table 1 Exome vs. Custom Enrichment 2
Table 2 TruSeq Enrichment Acronyms 9
Table 3 Sample Sheet Fields. 17
Table 4 TruSeq Enrichment Kit Sample Configurations 18
Table 5 User-Supplied Consumables 28
Table 6 User-Supplied Equipment 29
Table 7 Exome vs. Custom Enrichment 32
Table 8 DNA Libraries for Enrichment. 35
Table 9 Illumina General Contact Information. 79
Table 10 Illumina Customer Support Telephone Numbers 79

Overview

Chapter 1

Introduction 2
Enrichment Workflow 4

Introduction

This protocol explains how to capture exome or custom sequences of a human DNA library that was prepared using the 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:

- Simplest and most 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
- Most 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 the TruSeq DNA Sample Preparation Kit to provide convenient end-to-end enrichment sequencing solution
- Highest efficiency enrichment sequencing

  Most comprehensive enrichment coverage, highest uniformity, and lowest DNA input requirement

Whether you are performing exome or custom enrichment, the protocol is the same with the following exceptions that are 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 or 96 sample	24 or 96 sample

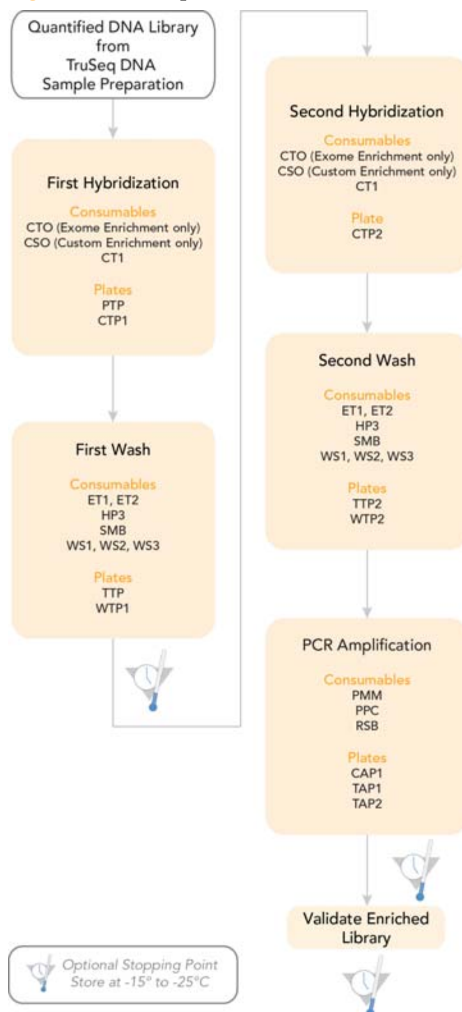
NOTE

In order to prepare libraries for enrichment, reference the Illumina *TruSeq DNA Sample Preparation Guide*. However, you must incorporate modifications to the protocol described in *TruSeq DNA Sample Prep* on page 33 of this guide.

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 the 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 the capture probes of target 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 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

Illumina recommends performing a quality assessment after running Gerald alignment using the TruSeq scripts to calculate metrics used to evaluate your results.

Getting Started

Chapter 2

Introduction 8
Acronyms 9
Best Practices 10
DNA Input Recommendations 14
Tracking Tools 16
Kit Contents 18
Consumables and Equipment 28

Introduction

This chapter 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 enrichment protocol described in the rest of this guide assumes that you are familiar with the contents of this chapter, have implemented all the recommendations, and have obtained all of the requisite equipment and consumables.

Acronyms

Table 2 TruSeq Enrichment Acronyms

Acronym	Definition	Acronym	Definition
CAP	Capture Target Plate	PPC	PCR Primer Cocktail
CSO	Custom Selected Oligos	PTP	Pooled Target Plate
CT1	Capture Target Buffer 1	RSB	Resuspension Buffer
CTO	Capture Target Oligos	SMB	Streptavidin Magnetic Beads
CTP	Capture Target Plate	TAP	Target Amplification Plate
dsDNA	Double-stranded DNA	TTP	Temporary Target Plate
ET1	Elute Target Buffer 1	WS1	Wash Solution 1
ET2	Elute Target Buffer 2	WS2	Wash Solution 2
HP3	2N NaOH	WS3	Wash Solution 3
PCR	Polymerase Chain Reaction	WTP	Wash Target Plate
PMM	PCR Master Mix, Polymerase		

Best Practices

When preparing genomic DNA libraries for sequencing, you should always adhere to good molecular biology practices.

Liquid Handling

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 or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer.
- If small volumes are unavoidable, then use due diligence to ensure that pipettes are correctly calibrated.
- Ensure 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, you pipette once from the reagent tubes with a larger volume, rather than many times with 1 µl volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

AMPure XP Handling

Follow appropriate handling methods when working with Agencourt AMPure XP Beads:

NOTE
Cleanup procedures have only been verified using a 96-well PCR or MIDI plate. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats.

NOTE
Cleanup procedures have only been tested and validated using the magnetic stand specified in this guide. Comparable performance is not guaranteed when using other magnets.

- Prior to use, allow the beads to come to room temperature.
- 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 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.
- For the wash steps, 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 may 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 it impacts the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time may be required.

- Use the Resuspension Buffer (RSB) for DNA elution.
- To maximize elution of the DNA, incubate the DNA/bead mixture 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 PMM 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 SMB tubes contain metallic particles that will quickly settle to the bottom of the tube. When aliquoting SMB particles, care should be taken to fully and completely mix the SMB 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 may 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 may 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 may 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.

DNA Input Recommendations

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 (Figure 2) or gel-free (Figure 3) method when run on a High Sensitivity DNA chip.

Figure 2 Gel Library on High Sensitivity DNA Chip

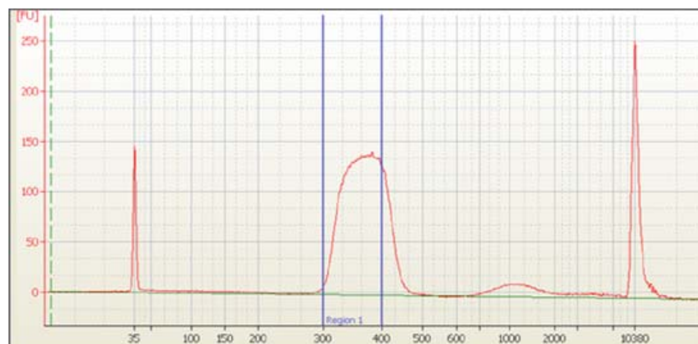
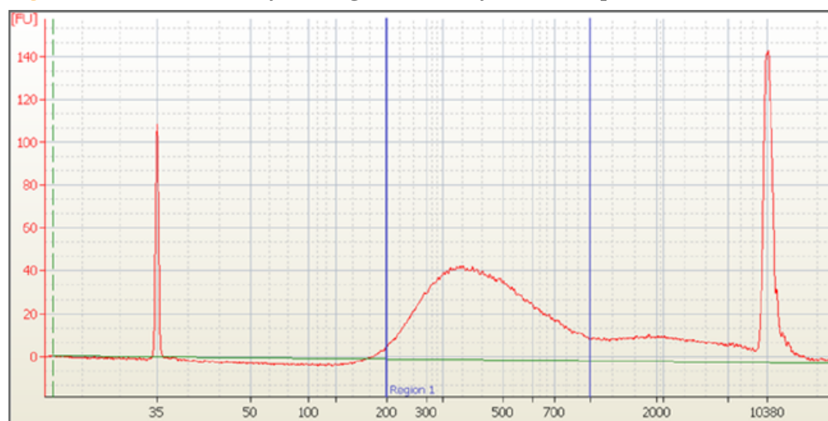


Figure 3 Gel-free Library on High Sensitivity DNA Chip



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 Cards** to guide you through the protocol, but with less detail than provided in this user guide.
- **Lab Tracking Forms** to record lab equipment start and stop times and record the barcode of each reagent and plate used in the protocol.
- **Sample Sheet Template** to record information about your samples for later use in data analysis.

NOTE
All of these documents can be downloaded via http://www.illumina.com/support/documentation.ilmn.

Lab Tracking Form

Create a copy of the lab tracking form for each run. Use it to track information about your sample preparation such as operator information, start and stop times, reagent lot numbers, and barcodes. This form can be filled out and saved online or printed and filled in by hand.

Sample Sheet

The sample sheet is a file that describes the samples in each lane, including the indexes used, and is required for demultiplexing following sequencing. For instructions on using the sample sheet to direct demultiplexing, see the analysis pipeline documentation.

The sample sheet is a comma-separated values (*.csv) file that contains the sample name and related information. Create the sample sheet using Excel or another text editing tool that supports .csv files. Fill in your sample sheet according to the guidelines provided in this section.

Include lanes with multiplexed samples in the sample sheet, listing the information below. Lanes with a single sample can be left out of the sample sheet. These single-sample lanes can then be aligned to a reference genome as specified in the CASAVA config.template.txt file.

Figure 4 Example: Sample Sheet

	A	B	C	D	E	F	G	H	I
1	FCID	Lane	SampleID	SampleRef	Index	Description	Control	Recipe	Operator
2	FC612PV	1	sample1	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
3	FC612PV	1	sample2	E_coli	TAGCTT	desc1	N	R1	j. doe
4	FC612PV	2	sample3	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
5	FC612PV	2	sample4	CMV	CGATGT	desc1	N	R1	j. doe
6	FC612PV	2	sample5	E_coli	CTTGTA	desc1	N	R1	j. doe
7	FC612PV	3	sample6	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
8	FC612PV	3	sample7	CMV	CGATGT	desc1	N	R1	j. doe
9	FC612PV	3	sample8	E_coli	CTTGTA	desc1	N	R1	j. doe
10	FC612PV	4	sample9	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
11	FC612PV	4	sample10	CMV	CGATGT	desc1	N	R1	j. doe
12	FC612PV	4	sample11	E_coli	CTTGTA	desc1	N	R1	j. doe
13	FC612PV	5	sample12	phi	TTAGGC	desc1	N	R1	j. doe
14	FC612PV	6	sample13	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
15	FC612PV	6	sample14	CMV	CGATGT	desc1	N	R1	j. doe
16	FC612PV	6	sample15	E_coli	CTTGTA	desc1	Y	R1	j. doe
17	FC612PV	7	sample16	Potato	ATCACG	desc1	Y	R1	j. doe
18	FC612PV	7	sample17	CMV	CGATGT	desc1	Y	R1	j. doe
19	FC612PV	7	sample18	CMV	TGACCA	desc1	Y	R1	j. doe

SampleSheet (2)

The sample sheet has the following fields:

Table 3 Sample Sheet Fields

Column Header	Description
FCID	The flow cell ID
Lane	A positive integer indicating the lane number (1–8)
Sample ID	The sample ID. This can be used to specify samples in the CASAVA config.template.txt file.
Sample Ref	The reference sequence for the sample. This can be used to specify a reference genome in the CASAVA config.template.txt.
Index	The index sequence
Description	A sample description
Control	Y indicates the lane is a control lane N indicates a sample
Recipe	The recipe used during sequencing
Operator	The name or ID of the operator

CAUTION

To avoid misidentifying samples, ensure that the sample IDs entered in the sample sheet correctly correspond to the DNA samples used.

Kit Contents

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

There are various kit sizes for each protocol to support various numbers of samples. Exome kits contain two boxes and Custom kits contain three boxes.

Table 4 TruSeq Enrichment Kit Sample Configurations

Number of Samples Supported	Exome	Custom
x8	•	
x24		•
x96	•	•

TruSeq Exome Enrichment Kit

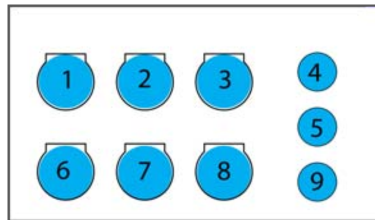
The TruSeq Exome Enrichment kits come in 8-sample and 96-sample kit box configurations.

8-Sample - Contents, 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 5 TruSeq Exome Enrichment Kit, 8 Sample, Box 1, part # 15017727



1–2 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927

3 Wash Solution 1 (TC#-WS1), part # 15015775

4 Resuspension Buffer (TC#-RSB), part # 15018075

5 Elute Target Buffer 2 (TC#-ET2), part # 15013008

6 Wash Solution 1 (TC#-WS1), part # 15015775

7 Wash Solution 3 (TC#-WS3), part # 15015933

8 Wash Solution 3 (TC#-WS3), part # 15015933

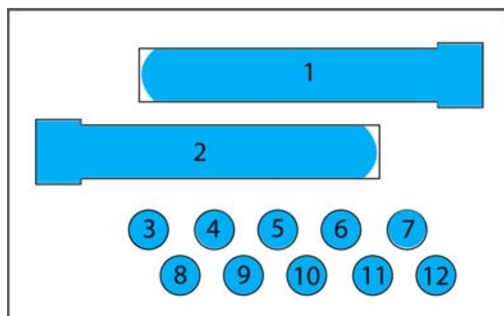
9 Elute Target Buffer 2 (TC#-ET2), part # 15013008

8-Sample - Contents, 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 6 TruSeq Exome Enrichment Kit, 8 Sample, Box 2, part # 15017728



1–2 Wash Solution 2 (TC#-WS2), part # 15015898

3–4 Capture Target Buffer 1 (TC#-CT1), part # 15015770

5–6 Elute Target Buffer 1 (TC#-ET1), part # 15013006

7–8 Capture Target Oligos (TC#-CTO), part # 15013000

9–10 2N NaOH (GA#-HP3), part # 11324596

11 PCR Master Mix, Polymerase (TC#-PMM), part # 15017038

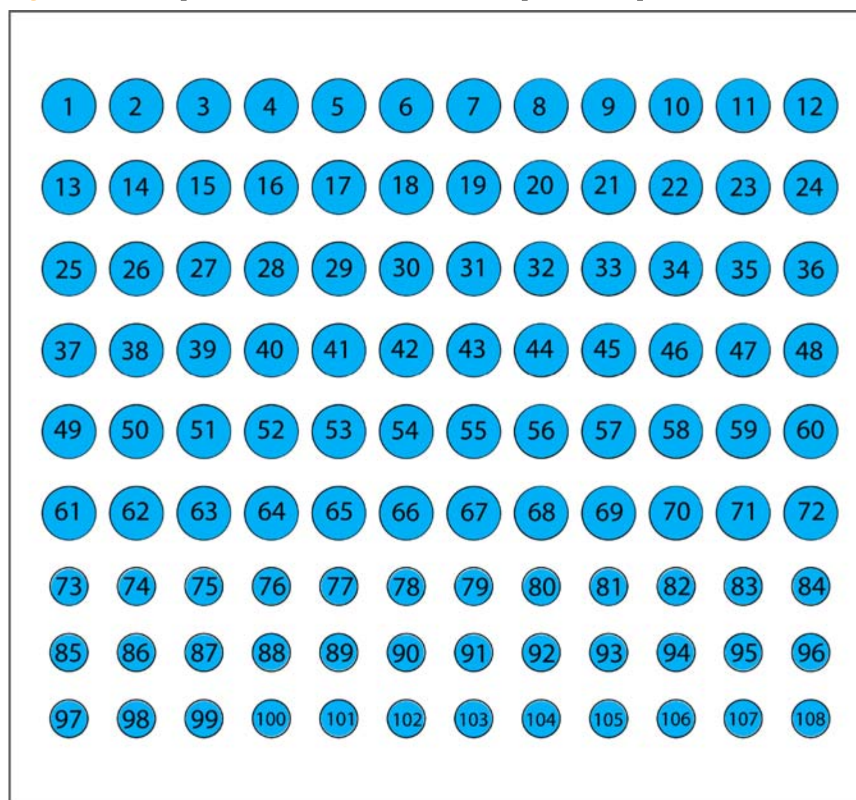
12 PCR Primer Cocktail (TC#-PPC), part # 15021793

96-Sample - Contents, 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 7 TruSeq Exome Enrichment Kit, 96 Sample, Box 1, part # 15019321



1–24 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927

25–48 Wash Solution 1 (TC#-WS1), part # 15015775

49–72 Wash Solution 3 (TC#-WS3), part # 15015933

73–96 Elute Target Buffer 2 (TC#-ET2), part # 15013008

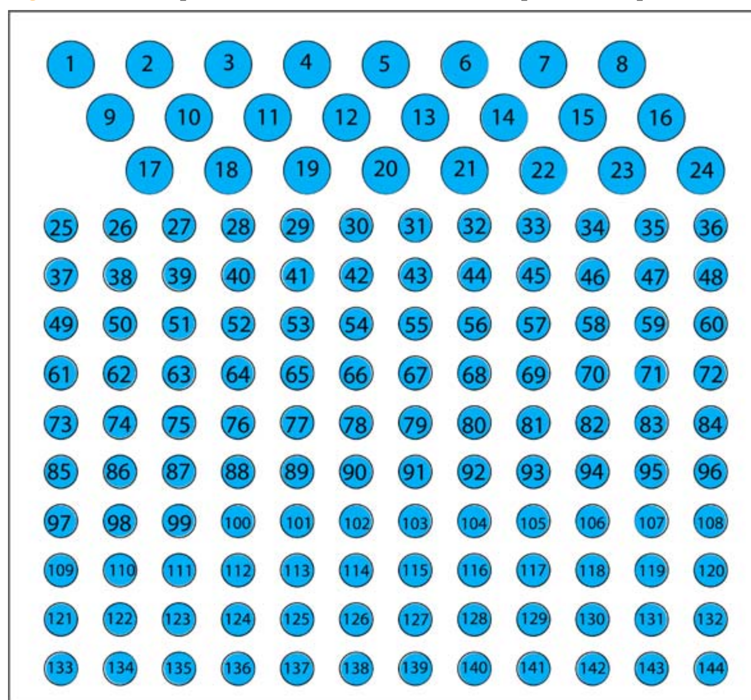
97–108 Resuspension Buffer (TC#-RSB), part # 15018075

96-Sample - Contents, 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 8 TruSeq Exome Enrichment Kit, 96 Sample, Box 2, part # 15019322



1–24 Wash Solution 2 (TC#-WS2), part # 15015898

25–48 Capture Target Buffer 1 (TC#-CT1), part # 15015770

49–72 Elute Target Buffer 1 (TC#-ET1), part # 15013006

73–96 Capture Target Oligos (TC#-CTO), part # 15013000

97–120 2N NaOH (GA#-HP3), part # 11324596

121–132 PCR Master Mix, Polymerase (TC#-PMM), part # 15017038

133–144 PCR Primer Cocktail (TC#-PPC), part # 15021793

TruSeq Custom Enrichment Kit

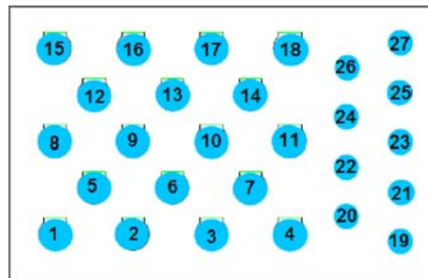
The TruSeq Custom Enrichment kits come in 24-sample and 96-sample kit box configurations.

24-Sample - Contents, 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 9 TruSeq Custom Enrichment Kit, 24 Sample, Box 1, part # 15022030



1–6 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927

7–12 Wash Solution 1 (TC#-WS1), part # 15015775

13–18 Wash Solution 3 (TC#-WS3), part # 15015933

19–24 Elute Target Buffer 2 (TC#-ET2), part # 15013008

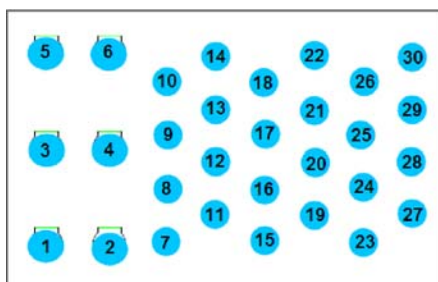
25–27 Resuspension Buffer (TC#-RSB), part # 15018075

24-Sample - Contents, 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 10 TruSeq Custom Enrichment Kit, 24 Sample, Box 2, part # 15022031



1–6 Wash Solution 2 (TC#-WS2), part # 15015898

7–12 Capture Target Buffer 1 (TC#-CT1), part # 15015770

13–18 Elute Target Buffer 1 (TC#-ET1), part # 15013006

19–24 2N NaOH (GA#-HP3), part # 11324596

25–27 PCR Master Mix, Polymerase (TC#-PMM), part # 15017038

28–30 PCR Primer Cocktail (TC#-PPC), part # 15021793

24-Sample - Contents, Box 3

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.

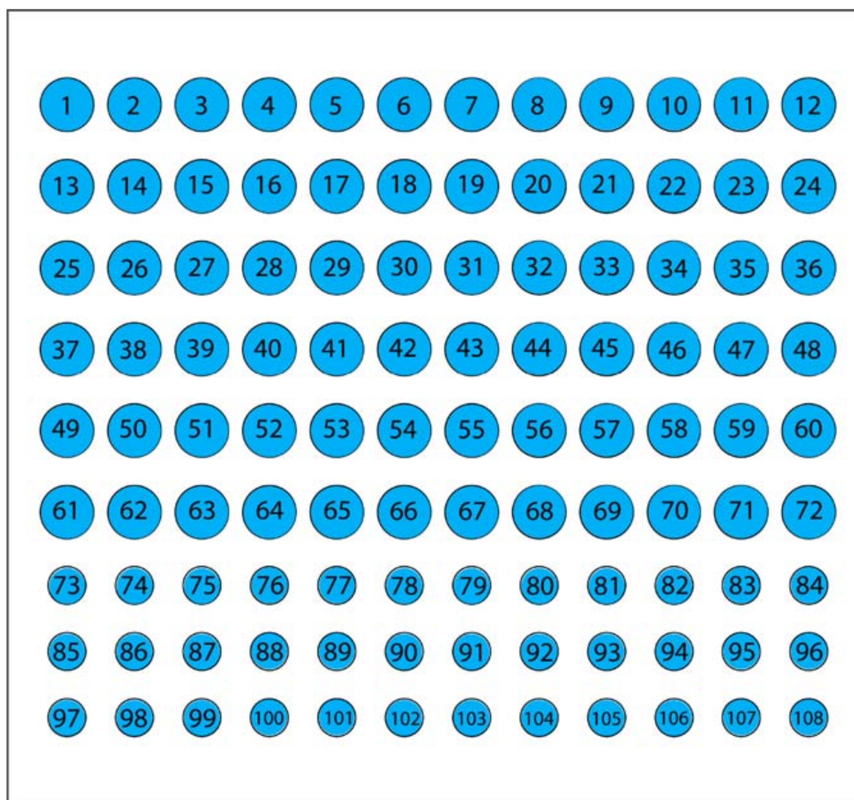
This box, part # 15022752, contains Custom Target Oligos (TC#-CSO), part # 15022753 and a lot number specific to each customer order. The boxes are configured for 6, 24, 120, and 240 slots.

96-Sample - Contents, 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 11 TruSeq Custom Enrichment Kit, 96 Sample, Box 1, part # 15022163



1–24 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927

25–48 Wash Solution 1 (TC#-WS1), part # 15015775

49–72 Wash Solution 3 (TC#-WS3), part # 15015933

73–96 Elute Target Buffer 2 (TC#-ET2), part # 15013008

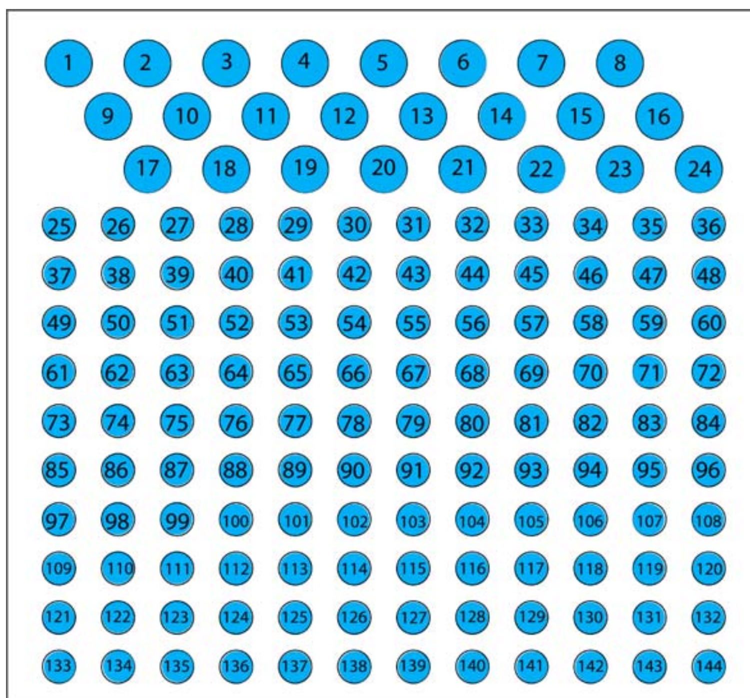
97–108 Resuspension Buffer (TC#-RSB), part # 15018075

96-Sample - Contents, 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 12 TruSeq Custom Enrichment Kit, 96 Sample, Box 2, part # 15022169



1–24 Wash Solution 2 (TC#-WS2), part # 15015898

25–49 Capture Target Buffer 1 (TC#-CT1), part # 15015770

50–74 Elute Target Buffer 1 (TC#-ET1), part # 15013006

75–99 2N NaOH (GA#-HP3), part # 11324596

100–112 PCR Master Mix, Polymerase (TC#-PMM), part # 15017038

113–125 PCR Primer Cocktail (TC#-PPC), part # 15021793

126–144 Empty

96-Sample - Contents, Box 3

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.

Consumables and Equipment

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

Table 5 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
96-well storage plates, round well, 0.8ml (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
PCR grade water	General lab supplier
PCR tubes	General lab supplier

Table 5 User-Supplied Consumables (Continued)

Consumable	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 6 User-Supplied Equipment

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
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
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

Enrichment Protocol

Chapter 3

Introduction 32
TruSeq DNA Sample Prep 33
First Hybridization 34
First Wash 37
Second Hybridization. 43
Second Wash. 45
PCR Amplification 51
Enriched Library Validation 55

Introduction

This chapter describes the TruSeq Enrichment protocol. Whether you are performing exome or custom enrichment, the protocol is the same with the following exceptions that are called out in the procedures:

Table 7 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 or 96 sample	24 or 96 sample

Follow the protocol in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation.

TruSeq DNA Sample Prep

Prior to TruSeq Enrichment, you must first prepare a human DNA library using the TruSeq DNA Sample Prep kit (see the Illumina *TruSeq DNA Sample Preparation Guide*). The 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 34 of this guide.

First Hybridization

This process mixes the DNA library with capture probes of targeted regions. The recommended hybridization time ensures 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.

Illumina-Supplied Consumables

- Custom Selected Oligos (CSO) (For Custom Enrichment Only)
- Capture Target Oligos (CTO) (For Exome Enrichment Only)
- Capture Target Buffer 1 (CT1)
- CTP1 (Capture Target Plate 1) barcode label

User-Supplied Consumables

- 300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate
- Adhesive Seal Roller
- Microseal 'B' Adhesive Seal
- PCR Grade Water
- 500 ng per DNA Library output from the Illumina TruSeq DNA Sample Prep Kit

Preparation

- 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 95°C for 10 minutes
  - b 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle
  - c 58°C for forever
- Apply a CTP1 barcode label to a new 300 µl 96-well PCR plate or twin.tech 96-well PCR plate.

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 15). 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)
•	•	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. Vortex the Capture Target Buffer 1 tube for 5 seconds. Visually ensure 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 with the CTP1 barcode. 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.

Reagent	Volume (µl)
For Custom Enrichment use Custom Selected Oligos For Exome Enrichment use Capture Target Oligos	10
Capture Target Buffer 1	50
Diluted DNA library	40
Total Volume per Sample	**100**

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

Incubate CTP1

1. Centrifuge the CTP1 plate to 280 xg for 1 minute.
2. Incubate the CTP1 plate on the pre-programmed 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 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.

Illumina-Supplied Consumables

- Elute Target Buffer 1 (ET1)
- Elute Target Buffer 2 (ET2)
- 2N NaOH (HP3)
- Streptavidin Magnetic Beads (SMB)
- Wash Solution 1 (WS1)
- Wash Solution 2 (WS2)
- Wash Solution 3 (WS3)
- TTP (Temporary Target) barcode label
- WTP1 (Wash Target Plate 1) barcode label

User-Supplied Consumables

- 96-well MIDI Plate
- 300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plates (2)
- Adhesive Seal Roller
- Microseal 'B' Adhesive Seals (7)
- PCR Tubes
- PCR Grade Water

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.
- Apply a TTP barcode label to a new 300 µl 96-well PCR plate or twin.tech 96-well PCR plate.
- Apply a WTP1 barcode label to a new 300 µl 96-well MIDI plate.
- [Optional] Label one new PCR tube per sample "First Elution for qPCR".

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 with the WTP1 barcode. Change the tip after each sample.
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. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
12. Remove the WTP1 plate from the magnetic stand.

Wash 1 WTP1 and Wash 2 WTP1

1. Vortex the Wash Solution 1 tube for 5 seconds. Visually ensure 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 ensure the beads are fully resuspended. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
5. Remove the WTP1 plate from the magnetic stand.

WS2 Clean Up

1. Vortex the Wash Solution 2 tube for 5 seconds. Visually ensure 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. Ensure that the beads are fully resuspended. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
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. Ensure that the beads are fully resuspended. Change the tip after each sample.
7. Transfer the entire contents of each well of the WTP1 plate to the corresponding well of a new 300 µl 96-well PCR plate with no barcode label. Change the tip after each sample.
8. Seal the PCR plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure the seal is secured.
9. Incubate the PCR 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 PCR 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 PCR plate.

13 Immediately remove and discard all of the supernatant from each well. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

14 Remove the PCR plate from the magnetic stand.

15 Add 200 µl Wash Solution 2 to each sample well of the PCR plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended. Change the tip after each sample.

16 Repeat steps 8–13 once.

Wash 3 WTP1

1 Remove the PCR plate from the magnetic stand.

2 Add 200 µl Wash Solution 3 to each well of the PCR plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly. Change the tip after each sample.

3 Place the PCR 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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

5 Repeat steps 1–4 once.

6 To remove any residual Wash Solution 3, seal the PCR plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure the seal is secured.

7 Briefly centrifuge the PCR plate to collect any residual Wash Solution 3.

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

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

10 Remove and discard any residual supernatant from each well. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

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

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

2 Remove the PCR plate from magnetic stand

3 Add 30 µl of the elution pre-mix to each well of the PCR plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Ensure that the beads are fully resuspended. Change the tip after each sample.

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

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

6 Centrifuge the PCR plate to 280 xg for 1 minute.

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

8 Carefully remove the adhesive seal from the PCR plate to avoid spilling the contents of the wells.

9 Transfer 29 µl of supernatant from each well of the PCR plate to the corresponding well of the new 96-well PCR plate labeled with the TTP barcode. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

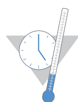
10 Add 5 µl Elute Target Buffer 2 to each well of the TTP plate containing samples to neutralize the elution. Gently pipette the entire volume up and down 10–20 times to mix thoroughly. Change the tip after each sample.

11 Seal the TTP plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure 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* immediately, the protocol can be safely stopped here. If you are stopping, seal the TTP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days. When proceeding, thaw the PCR 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 ensures the targeted regions are further enriched.

Illumina-Supplied Consumables

- Custom Selected Oligos (CSO) (For Custom Enrichment Only)
- Capture Target Oligos (CTO) (For Exome Enrichment Only)
- Capture Target Buffer 1 (CT1)
- CTP2 (Capture Target Plate 2) barcode label

User-Supplied Consumables

- 300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate
- First Elution
- Microseal 'B' Adhesive Seal
- PCR Grade Water

Preparation

- 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 First Elution plate from -15° to -25°C storage, if it was stored at the conclusion of *First Wash* and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed First Elution plate to 280 xg for 1 minute
  - Remove the adhesive seal from the thawed First Elution 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
- Apply a CTP2 barcode label to a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate

Make CTP2

1. Vortex the Capture Target Buffer 1 tube for 5 seconds. Visually ensure 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 a new 300 µl 96-well PCR plate labeled with a CTP2 barcode. 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	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 ensure the seal is secured.

Incubate CTP2

1. Centrifuge the CTP2 plate to 280 xg for 1 minute.
2. Incubate the CTP2 plate on the pre-programmed 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 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 37.

Illumina-Supplied Consumables

- Elute Target Buffer 1 (ET1)
- Elute Target Buffer 2 (ET2)
- 2N NaOH (HP3)
- Streptavidin Magnetic Beads (SMB)
- Wash Solution 1 (WS1)
- Wash Solution 2 (WS2)
- Wash Solution 3 (WS3)
- TTP2 (Temporary Target Plate 2) barcode label
- WTP2 (Wash Target Plate 2) barcode label

User-Supplied Consumables

- 96-well MIDI Plate
- 300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plates (2)
- Adhesive Seal Roller
- Microseal 'B' Adhesive Seals (7)
- PCR Tubes
- PCR Grade Water

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.

- Apply a TTP2 barcode label to a new 300 µl 96-well PCR plate or twin.tech 96-well PCR plate.
- Apply a WTP2 barcode label to a new 300 µl 96-well MIDI plate.
- [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 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 with the WTP2 barcode. Change the tip after each sample.
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. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
12. Remove the WTP2 plate from the magnetic stand.

Wash 1 WTP2 and Wash 2 WTP2

1. Vortex the Wash Solution 1 tube for 5 seconds. Visually ensure 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 ensure the beads are fully resuspended. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
5. Remove the WTP2 plate from the magnetic stand.

WS2 Clean Up

1. Vortex the Wash Solution 2 tube for 5 seconds. Visually ensure 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. Ensure that the beads are fully resuspended. Change the tip after each sample.
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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
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. Ensure that the beads are fully resuspended. Change the tip after each sample.

7 Transfer the entire contents of each well of the WTP2 plate to the corresponding well of a new 300 µl 96-well PCR plate with no barcode label. Change the tip after each sample.

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

9 Incubate the PCR plate in 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 PCR 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 PCR plate.

13 Immediately remove and discard all of the supernatant from each well. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

14 Remove the PCR plate from the magnetic stand.

15 Add 200 µl Wash Solution 2 to each sample well of the PCR plate. Gently pipette the entire volume up and down 10–20 times. Mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended. Change the tip after each sample.

16 Repeat steps 8–13 once.

Wash 3 WTP2

1 Remove the PCR plate from the magnetic stand.

2 Add 200 µl Wash Solution 3 to each well of the PCR plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly. Change the tip after each sample.

3 Place the PCR 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. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.
5. Repeat steps 1–4 once.
6. To remove any residual Wash Solution 3, seal the PCR plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure the seal is secured.
7. Briefly centrifuge the PCR plate to collect any residual Wash Solution 3.
8. Place the PCR plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.
9. Carefully remove the adhesive seal from the PCR plate to avoid spilling the contents of the wells.
10. Remove and discard any residual supernatant from each well. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

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

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

2. Remove the PCR plate from magnetic stand
3. Add 30 µl of the elution pre-mix to each well of the PCR plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Ensure that the beads are fully resuspended. Change the tip after each sample.
4. Seal the PCR plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure the seal is secured.
5. Let the PCR plate stand at room temperature for 5 minutes.

6 Centrifuge the PCR plate to 280 xg for 1 minute.

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

8 Carefully remove the adhesive seal from the PCR plate to avoid spilling the contents of the wells.

9 Transfer 29 µl of supernatant from each well of the PCR plate to the corresponding well of the new 96-well PCR plate labeled with the TTP2 barcode. Take care not to disturb the Streptavidin Magnetic Beads. Change the tip after each sample.

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. Change the tip after each sample.

11 Seal the TTP2 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and ensure the seal is secured.

12 [Optional] Dilute 2 µl of the second elution 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.

NOTE
The Second 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.

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.

Illumina-Supplied Consumables

- PCR Master Mix (PMM)
- PCR Primer Cocktail (PPC)
- Resuspension Buffer (RSB)
- CAP1 (Cleaned Amplification Plate 1) barcode label
- TAP1 (Target Amplification Plate 1) barcode label

User-Supplied Consumables

- 300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plates (2)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals (2)

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 *AMPure XP Handling* on page 11.
- 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 98°C for 30 seconds
  - b 10 cycles of:
    98°C for 10 seconds
    60°C for 30 seconds
    72°C for 30 seconds
  - c 72°C for 5 minutes
  - d Hold at 10°C
- Apply CAP1 and TAP1 barcode labels to 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate.

Make TAP1

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

Reagent	Volume (µl)
Second Elution	20
PCR Master Mix	25
PCR Primer Cocktail	5
Total Volume	**50**

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

3 Centrifuge the TAP1 plate to 280 xg for 1 minute.

Amp PCR

1 Amplify the TAP1 plate on the pre-programed thermal cycler as follows:
   a 98°C for 30 seconds
   b 10 cycles of:
     98°C for 10 seconds
     60°C for 30 seconds
     72°C for 30 seconds
   c 72°C for 5 minutes
   d Hold at 10°C

Make TAP2

NOTE
Before performing clean up, *AMPure XP Handling* on page 11 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. Change the tip after each sample.

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. Take care not to disturb the beads. Change the tip after each sample.

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. Change the tip after each sample.

7 Incubate the PCR plate for at least 30 seconds at room temperature, then remove and discard the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.

8 Repeat steps 6–7 once for a total of two 80% EtOH washes.

9 Let the TAP1 plate stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

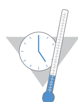
Make CAP1

1 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. Change the tip after each sample.

2 Incubate the TAP1 plate at room temperature for 2 minutes.

3 Place the TAP1 plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

4 Transfer 30 µl of the clear supernatant from each well of the TAP1 plate to the corresponding well of the new 300 µl 96-well PCR plate labeled with the CAP1 barcode. Change the tip after each sample.



SAFE STOPPING POINT

If you do not plan to proceed to *Enriched Library Validation* immediately, the protocol can be safely stopped here.If you are stopping, seal the CAP1 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.

Appendix A

Data Assessment

Introduction 58
Script Installation 59
Script Usage 60
Statistics 64
Coverage 70

Introduction

Illumina recommends, after running Gerald alignment, to run a stepwise data assessment using the TruSeq Enrichment scripts to calculate metrics used to evaluate your results.

NOTE
The TruSeq Enrichment script can be downloaded via http://www.illumina.com.

Script Installation

The *install.sh* script installs the TruSeq Enrichment scripts, tested with CASAVA 1.7.0 and CASAVA 1.8.0.

It performs the following operations:

1. Copy the .pl and .pm files into the destination directory
2. Replace some constants in the .pl, .pm, and config files with paths to the CASAVA perl libraries and the CASAVA executable, so the scripts can find the CASAVA components they depend on.

Syntax:

Use the following syntax to install the TruSeqEnrichment scripts for use with only one version of Casava:

./install.sh <casava_installation_dir> <destination_dir>

Use the following syntax for compatibility with Casava 1.7.0 and 1.8.0:

./install.sh <casava_1.7.0_dir> <casava_1.8.0_dir> <destination_dir>

Example: ./install.sh /opt/GOAT/CASAVA_1.8.0 ~/myscripts/TruSeqEnrichment1.2/

Script Usage

The TruSeqEnrichmentAnalysis.pl script generates a Makefile that can be invoked with make or, on a cluster, qmake. It can run either as a post-run command or as a stand-alone command. Multiple processors/CPUs are supported. If you configure N TruSeq Enrichment runs on the command line (with multiple --lanes options), the Makefile can take advantage of up to 2*N CPUs or cores.

There are three steps to the analysis:

Steps 1 and 2 can be executed concurrently, while step 3 must wait for steps 1 and 2 to complete. This logic is built into the Makefiles.

1 Post-alignment statistics
2 CASAVA-build
3 Post-CASAVA statistics.

NOTE

Additional jobs will be generated by the CASAVA build independent of the number of jobs you tell make or qmake to use (with the -j option). The number of jobs generated by the CASAVA build can be controlled by passing the standard CASAVA command line parameters to TruSeqEnrichmentAnalyses.pl script. For example, --jobsLimit=8.

The following is an example of how to use the TruSeq Enrichment quality assessment scripts with CASAVA 1.8:

```
cd ~/mydata/FC123/Aligned

perl ~/myscripts/TruSeqEnrichment1.0/TruSeqEnrichmentAnalysis.pl \

--TruSeqEnrichmentRunId=FC123 \

--project proj \

--sgeAuto \

--sgeQueue=my.q \

--jobsLimit=4
```

The following is an example of how to use the TruSeq Enrichment quality assessment scripts with CASAVA 1.7:

```
cd ~/mydata/FC123/GeraldResults
```

```
perl ~/myscripts/TruSeqEnrichment1.0/TruSeqEnrichmentAnalysis.pl \
--TruSeqEnrichmentRunId=FC123 \
--lanes=1,2 \
--lanes=3,4 \
--refSequences= /iGenomes/Homo_sapiens/UCSC/hg19/Sequence/Chromosomes  \
--sgeAuto \
--sgeQueue=my.q \
--jobsLimit=4
```

For TruSeq Custom (both CASAVA 1.7 and CASAVA 1.8), include the options --targetsDir and -targetsName. Together these two options specify the location of the targeted_regions files provided by Illumina with your custom probe set. For example, for the targeted regions file at this location:

/illumina/mydata/custom/projectABC_targeted_regions.txt

Add the following options:

```
--targetsDir /illumina/mydata/custom \
--targetsName projectABC
```

A detailed usage message is printed out when the script is run with the --help option.

Usage: TruSeqEnrichmentAnalysis.pl [options]:

--**targetsName**–Name of targeted regions (default: TruSeq_exome)

--**targetsDir**–Directory where the targeted regions file (<targetsName>_targeted_regions.txt) is located (default: TruSeqEnrichment scripts directory)

--**TruSeqEnrichmentProjectDir**–Directory for results (default: <exportDir>/TrueSeqEnrichment or <alignmentDir>/TruSeqEnrichment)

--**TruSeqEnrichmentRunId**–User-specifed ID for TruSeq analysis (required for CASAVA 1.7. Default for CASAVA 1.8 is the project name)

--**casavaProjectDir**–Directory for CASAVA build (uses pre-existing build) (default: <exportDir>/casava_build)

--**overwrite**–Proceed even if TruSeqEnrichmentProjectDir exists. Old files will not be deleted; they may interfere with analysis

--**emailList=<address>** –Addresses to send email to when analysis is finished

--**help**–Print this help message

--**casavaParameters=<params>**–Additional parameters for CASAVA build. Enclose in ""

CASAVA 1.7 parameters:

--**lanes** –Comma-separated list of the lanes to use. Lanes will be combined (required for CASAVA 1.7)

--**exportDir** –GERALD Directory containing export.txt files (default: current directory)

CASAVA 1.8 parameters:

example: --alignmentDir Alignments --project myProject

example: --alignmentDir Alignments --project myProject --samples samp1,samp2,samp3

--**alignmentDir**–Directory containing CASAVA 1.8 alignments (called Aligned by default). Default: current directory

--**project <ProjectName>**–Project name (omit the Project_ prefix). Required for CASAVA 1.8

--**sample <SampleName(s)>**–comma-separated list of samples to analyze (omit the Sample_ prefix) (default: all samples in project)

Parameters passed to CASAVA:

--**refSequences|ref**–PATH of the reference genome sequences (required for CASAVA 1.7, unless CASAVA build has already been done. For CASAVA 1.8, automatically extracted from alignment configuration)

--**workflowAuto|wa**–Passed to CASAVA. See *CASAVA Software User Guide* (either --workflowAutof or sgeAuto required if CASAVA build has not been done)

--**sgeAuto|sa**–Passed to CASAVA. See *CASAVA Software User Guide* (either --workflowAutof or sgeAuto required if CASAVA build has not been done)

--**queue|sgeQueue** –Passed to CASAVA. Name of SGE queue, if the sgeAuto workflow is used (default: all.q)

--**jobsLimit|j** –Make number of jobs for CASAVA to run at once

NOTE

- If CASAVA1.8 is used for alignment, then it must also be used for TruSeqEnrichmentAnalysis. The same is true of CASAVA 1.7.
- Additional CASAVA parameters can be passed in with the -casavaParameters option.
- This script generates a Makefile in <TruSeqEnrichmentProjectDir>. It can be run with make or submitted to the SGE with qmake. Each TruSeq Enrichment run (determined by the number of times --lanes is specified) will generate a CASAVA build. The number of processors used in each CASAVA build is determined by the standard CASAVA command line parameters: for example, --jobsLimit=4.

NOTE

For more examples, reference the Readme file in the sample data set that can be downloaded available via http://www.illumina.com.

Statistics

The TruSeq Enrichment quality assessment script calculates two sets of statistics; post-alignment and post-CASAVA. The post-alignment statistics are derived from the Gerald s_*_export.txt output files and count the number of reads that overlap with each targeted region. The post-CASAVA statistics are derived from the CASAVA build and calculate the coverage at each base within a region.

For example, for a 300 bp region with 6 unique 100 bp reads entirely contained inside the region, the readcounts would be 6 and the average coverage would be 6*100/300 = 2X.

For paired end runs, the CASAVA removes duplicates by default (--rmDup=YES), meaning that CASAVA removes all the read pairs with the same start and end positions and strand. This can be overridden on the command line with casavaParameters= "--rmDup=NO".

The directory structure of the output is as follows:

<TruSeqEnrichmentProjectId>

- <TruSeqEnrichmentRunId1>
- •
- •
- •
- <TruSeqEnrichmentRunIdN>

Each of the <TruSeqEnrichmentRunIdX> folders contains the output from one TruSeq Enrichment analysis. If multiple different --lanes options are specified on the command line and a single --TruSeqEnrichmentRunID is provided, then each analysis will be placed in a folder named <TruSeqEnrichmentRunID>_<lanes>. Alternatively, if different --TruSeqEnrichmentRunIDs are specified for each --lanes argument on the command line, then folders shown above will be named with the corresponding TruSeqEnrichmentRunId.

To combine data from multiple different runs in one analysis, use the same TruSeqEnrichmentRunId multiple times. For example:

```
--TruSeqEnrichmentRunID FC1234 --lanes 7 --exportDir dir1 \
--TruSeqEnrichmentRunID FC1234 --lanes 6,7,8 --exportDir dir2
```

The following options will produce two separate TruSeq Enrichment analyses (named FC1234_7 and FC1234_678):

```
--TruSeqEnrichmentRunID FC1234 \
```

```
--lanes 7 --exportDir dir1 \
--lanes 6,7,8 --exportDir dir2
```

The results of post-alignment analysis are in the top level of the <TruSeqEnrichmentRunIdX> folder, and the results of the post-CASAVA statistics are in the CASAVA_stats subfolder (<runIDx>/CASAVA_stats). There may also be a casava_ build folder in the <TruSeqEnrichmentRunIdX> folder. This is the default location for the CASAVA build, but it can be overridden on the command line.

Enrichment Statistics

The*<TruSeqEnrichmentRunId>_enrichment_statistics.txt* file contains the following enrichment statistics:

Run ID–TruSeqRunId, derived from the command line options.

Lane–Lanes used in this run.

Read Length–Read length of the sequencing run.

Targeted Regions–Name of the pool of probes used for enrichment, as given on the command line.

Pull-down Region Size (calculated on single probe regions)–An estimate of the size of the region pulled down by a single probe. It depends strongly on the size of the library used. See *Read Distribution* for details on how this is calculated.

Total Regions Size–Number of bases contained in the full set of targeted regions.

Mean Coverage Estimate (calculated from read counts)–An estimate of the coverage, calculated from the readcounts. The calculation is: (# of reads in targeted regions) * (Read length)/(Total regions size).

NOTE

The mean coverage estimate is calculated from the export.txt files prior to the duplicate pair removal. For more precise coverage numbers, see the post-CASAVA statistics.

Number of Reads (PF + Aligned)–Number of reads that pass filter and align.

Number of Reads in Targeted Regions–Number of reads that overlap the targeted region.

Enrichment in Targeted Regions–Percentage of reads (PF+aligned) that overlap the targeted region.

Enrichment in Targeted Regions +/-150 bp–Percentage of reads (PF+aligned) that overlap the targeted region +/- 150 bp.

Number of Targeted Regions–Total number of targeted regions in the pool of probes.

Number of Targeted Regions with No Reads–Number of targeted regions with 0 reads.

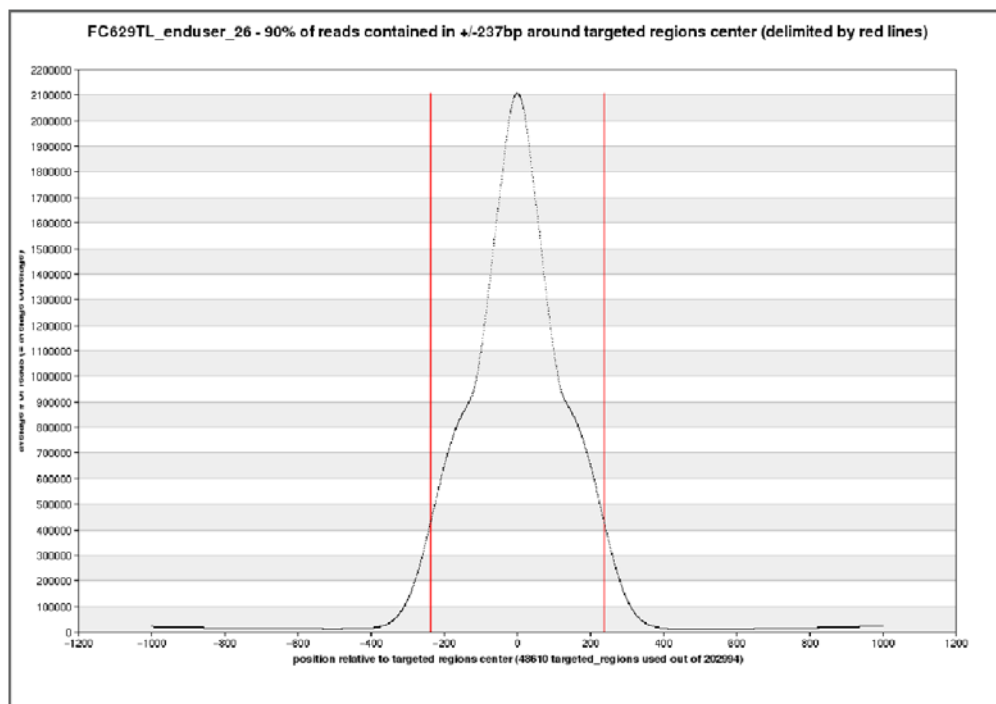
Percentage of Targeted Regions with No Reads–Percentage of targeted regions that contain 0 reads.

Read Distribution

The *<TruSeqEnrichmentRunId>_average_read_per_base_around_probes_center.png* file displays the distribution of reads around the center of the targeted regions. It is calculated as follows:

1. Identify short (<300 bp), isolated targeted regions, with isolated defined as the center of the region is at least 2000 bp away from center of the nearest neighboring region.
2. Plot a histogram of reads in these regions, where the x axis is the distance from the center of the region and the y axis is the number of reads (summed over all the regions) falling that distance from the center.
3. Determine the window around the region center which contains 90% of all the reads in a 2,000 bp window around the center. This 90% is configurable with the readsPercent command line option.

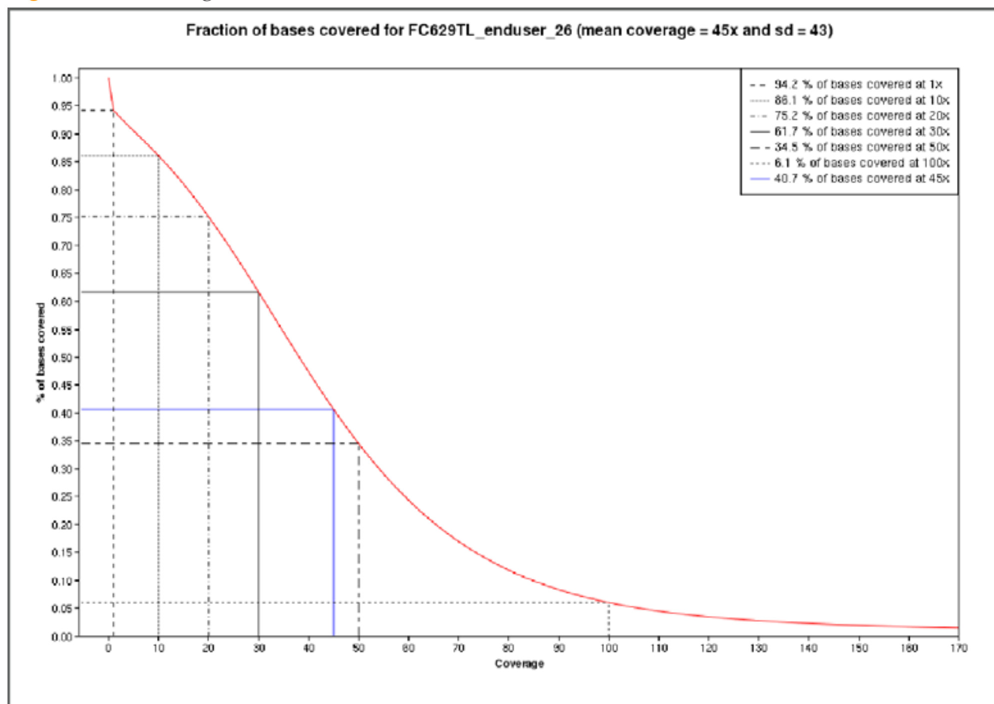
Figure 13 Read Distribution Plot



Coverage Level

In the *<TruSeqEnrichmentRunId>_fraction_bases_covered.png* file, the X axis shows coverage level, and the Y axis shows the percentage of targeted bases covered at that level or higher, allowing the identification of the fraction of targeted bases that are covered at a given coverage level. The lines on the plot and the corresponding data in the legend show the percentage of targeted bases covered at specific coverage levels. The coverage levels shown are: 1X, 10X, 20X, 30X, 50X, 100X, and mean coverage. This data is also available as text in the <TruSeqEnrichmentRunId>_coverage.txt file.

Figure 14 Coverage Level Plot

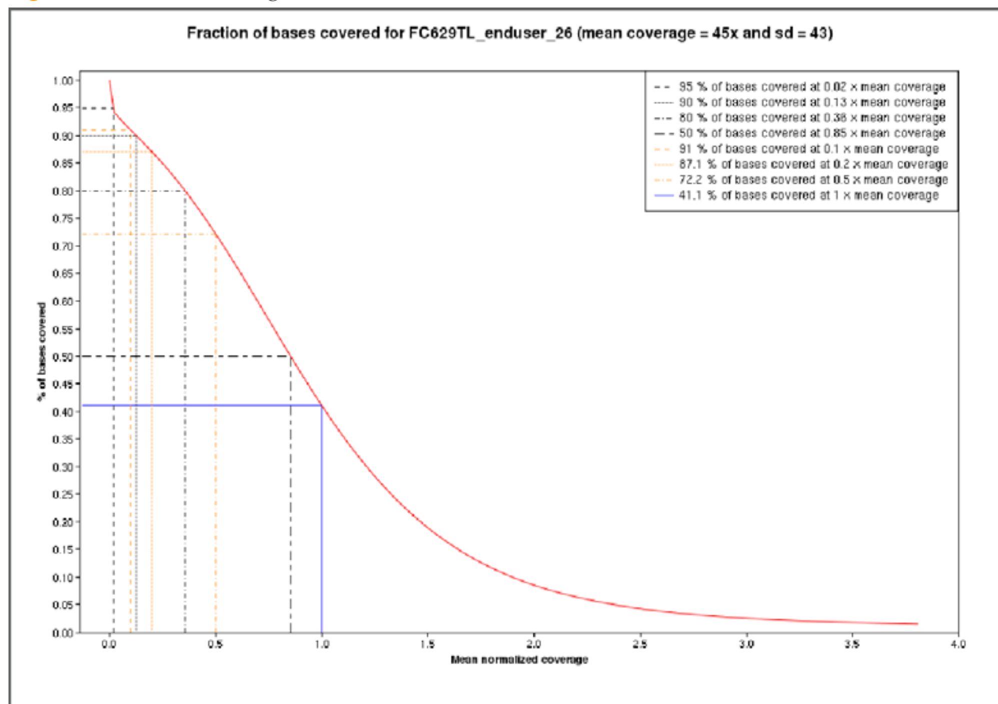


The coverage level at every base in the targeted regions is extracted from the CASAVA build. Statistics are then calculated on this data set: the mean and the percentage of bases at or above a given coverage level.

Mean Coverage Level

The *<TruSeqEnrichmentRunId>_fraction_bases_covered_mean_normalized.png* file is similar to the *<TruSeqEnrichmentRunId>_fraction_bases_covered.png* file, but the X axis is normalized to the mean coverage level. This provides the capability to compare data on the same scale from runs that have different mean coverage levels.

Figure 15 Mean Coverage Level Plot



As an example, if two runs have a substantially different number of total reads, the percentage of bases that are covered at 0.2 times the mean coverage level can be compared to better understand whether the coverage levels would be comparable if there were the same total number of reads for both samples.

Coverage

This <*TruSeqEnrichmentRunId*>*_coverage.txt* file contains four tables:

- The first table contains the same data shown in the legend of the plot <TruSeqEnrichmentRunId>_Fraction_bases_covered.png which displays the percentage of bases covered at or above 1X, 10X, 20X, 30X, 50X, 100X, and mean coverage.
- The second table is an inverse of the first table, displaying the coverage at a fixed percentage of bases (95%, 90%, 80%, 50%). For example, when it is desirable to cover at least 80% of the targeted bases at a 20X coverage level, the table is used to see that 80% of the targeted bases were covered at, for example, a 15X coverage level. This suggests that approximately 33% more sequence data is required to achieve a 20X coverage level.
- The third and fourth tables contain similar data for x mean normalized coverage.

Controls

Appendix B

Introduction 72
Sample-Independent Controls 73
Sample-Dependent Controls 74

Introduction

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 the *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 consisting of 150 probes has been included in the CTO probe pool, which functions as sample dependent controls for the enrichment process.

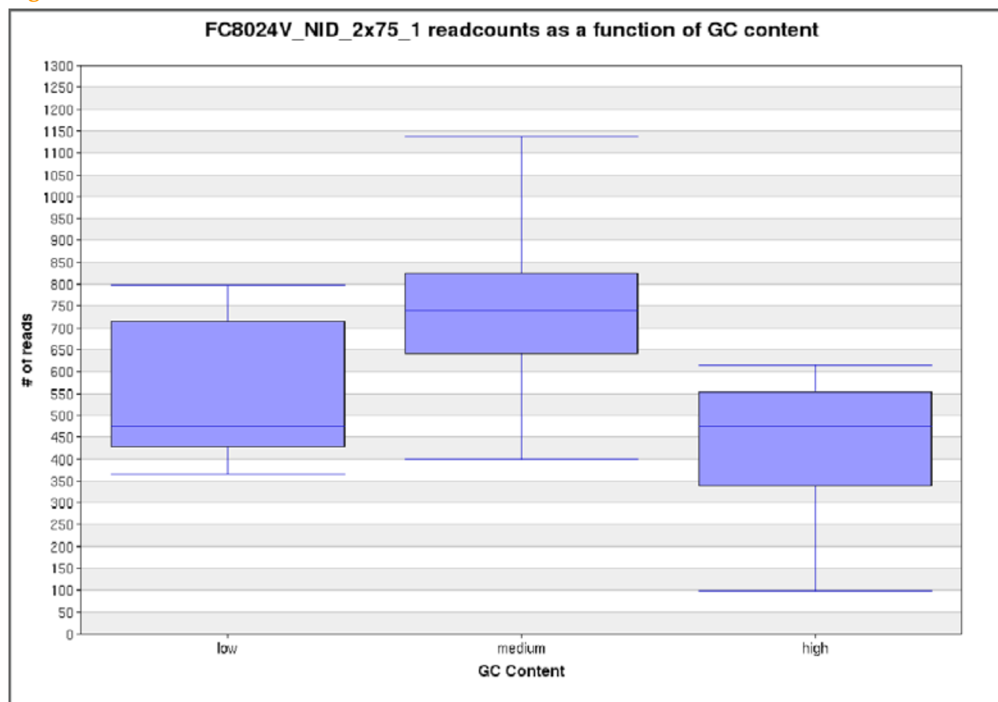
GC Control Probes

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



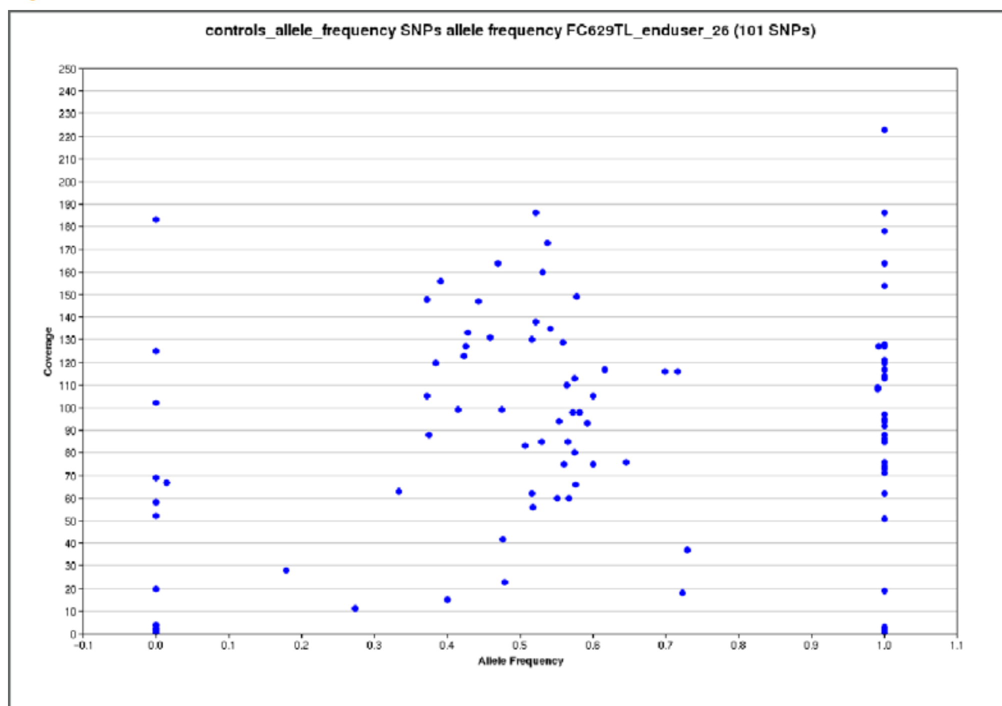
Each read that overlaps by at least one base with the probe region is counted. This data is derived directly from the export.txt files. Duplicates are not removed. For each stringency level, the readcounts for every probe at that stringency level is used to generate the box and whiskers. The box and 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 may reflect the ability to accurately call a SNP following enrichment 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

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to http://www.illumina.com/support/documentation.ilmn. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.

Illumina, Inc.
9885 Towne Centre Drive
San Diego, CA 92121-1975
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com