

TruSeq™ Exome Enrichment Guide

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Introduction

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

The exome enrichment protocol offers:

- ▶ Simplest and Most Scalable Workflow
 - Master-mixed reagents coupled with plate-based processing for up to 576 samples in one 96-well plate
- ▶ Most Cost-Effective Exome Sequencing
 - Pre-enrichment pooling of up to six samples
- ▶ Integrated Solution
 - Optimized for use with the TruSeq DNA Sample Preparation Kit to provide convenient end-to-end exome sequencing solution
- ▶ Highest Efficiency Exome Sequencing
 - Most comprehensive exome coverage, highest uniformity, and lowest DNA input requirement



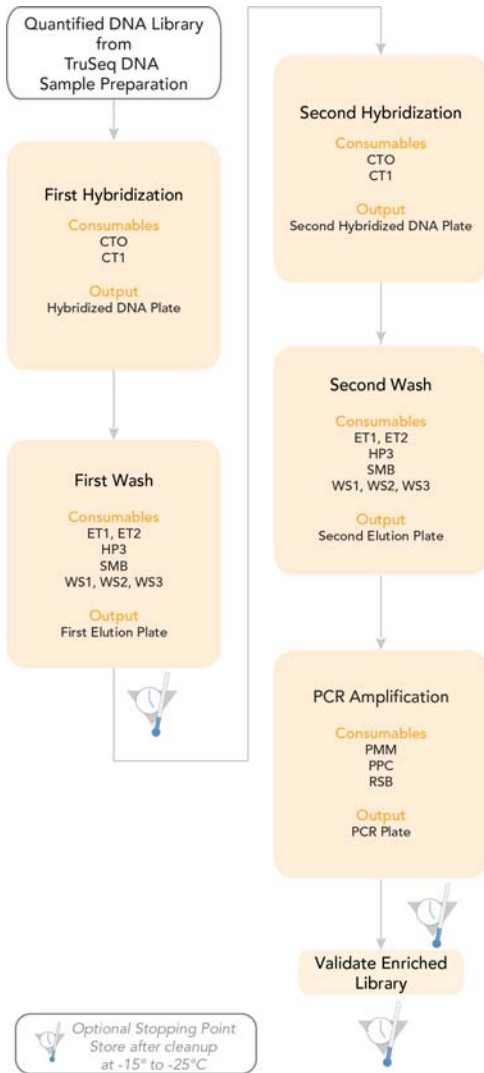
NOTE

In order to prepare libraries for exome 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 16.

Exome Enrichment Workflow

This section describes the TruSeq Exome Enrichment workflow.

Figure 1 TruSeq Exome Enrichment Workflow



TruSeq DNA Sample Prep

Prior to TruSeq Exome Enrichment, you must first prepare a human DNA library using the TruSeq DNA Sample Prep kit. However, in order to support TruSeq Exome Enrichment, you must excise a band from the gel spanning the width of the lane and ranging in size from 300–400 bp, rather than 400–500 bp required for TruSeq DNA Sample Preparation.

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.

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.

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.

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. It is similar to the First Wash procedure.

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.

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.

Acronyms

Table 1 TruSeq Exome Enrichment Acronyms

Acronym	Definition
CT1	Capture Target Buffer 1
CTO	Capture Target Oligos
dsDNA	Double-stranded DNA
ET1	Elute Target Buffer 1
ET2	Elute Target Buffer 2
HP3	2N NaOH
PCR	Polymerase Chain Reaction
PMM	PCR Master Mix, Polymerase
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
WS1	Wash Solution 1
WS2	Wash Solution 2
WS3	Wash Solution 3

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 ($\pm 0.5 \mu\text{l}$) can sometimes give rise to very large differences in cluster numbers ($\sim 100,000$). Small volume pipetting can also 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 ensure that pipettes are correctly calibrated. Also, care should be taken, because solutions of high molecular weight double-stranded (ds)DNA 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 $1 \mu\text{l}$ volumes. Prepare a master mix of enzymes, water, buffer, etc. and aliquot this in a single pipetting movement to individual samples to standardize across multiple samples.

AMPure XP Handling

The following indicates the appropriate handling methods when working with Agencourt AMPure XP Beads:

- ▶ 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.
- ▶ Change the tips for each sample or when using a multichannel pipette change the tips after each column.
- ▶ Let the mixed samples incubate for 5 minutes at room temperature for maximum binding.

- ▶ The minimum time required for the beads to be separated from the solution is 5 minutes, but may be longer depending on the volume of the reaction and on the magnetic stand used. Wait at least 5 minutes for the solution to clear before proceeding to the next step.
- ▶ 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. Eighty-percent 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.
- ▶ Remove the reaction plate from the magnetic stand and let it air-dry at room temperature. 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 for DNA elution. When processing samples, gently pipette up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
- ▶ To maximize elution of the DNA, incubate the DNA/bead mixture for 5 minutes at room temperature before placing the samples onto the magnet.

Target Capture

The following specifies recommended target capture methods during exome 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 Quantitation

Illumina recommends 500 ng per library for the TruSeq Exome Enrichment protocol. The ultimate success of exome enrichment strongly depends on using an accurately quantified amount of input DNA library. Therefore, the correct quantitation of the DNA library is essential.

Illumina strongly recommends quantifying the starting DNA library by qPCR. For more information on using qPCR to quantify libraries, reference the *Illumina qPCR Quantification Protocol Guide*.

Input DNA Library Quality

The DNA library quality is important for the success of the TruSeq Exome Enrichment assay. Illumina recommends using the 300–400 bp libraries from the Illumina TruSeq DNA Sample Preparation protocol as input into TruSeq Exome Enrichment. 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. If using the Agilent Bioanalyzer, we recommend loading 1 μ l of the input library (diluted 1:100) on an Agilent High Sensitivity DNA chip.

Kit Contents

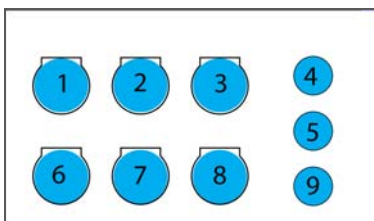
Check to ensure that you have all of the reagents identified in this section before proceeding to the exome enrichment procedures.

Kit 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 2 TruSeq Exome Enrichment Kit, Box 1, part # 15017727



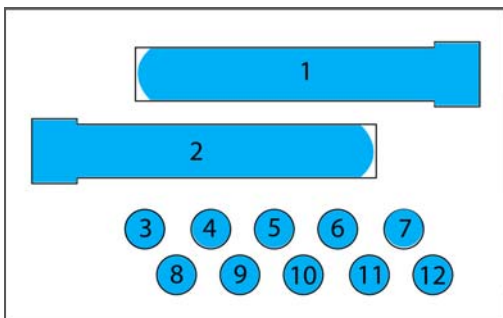
- 1 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927
- 2 Streptavidin Magnetic Beads (TC#-SMB), part # 15015927
- 3 Wash Solution 1 (TC#-WS1), part # 15015775
- 4 Resuspension Buffer (TC#-RSB), part # 15018075
- 5 Elute Target Buffer 2 (TC#-ET2), part # 15013008
- 6 Wash Solution 1 (TC#-WS1), part # 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

Kit 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 3 TruSeq Exome Enrichment Kit, Box 2, part # 15017728



- 1 Wash Solution 2 (TC#-WS2), part # 15015898
- 2 Wash Solution 2 (TC#-WS2), part # 15015898
- 3 Capture Target Buffer 1 (TC#-CT1), part # 15015770
- 4 Capture Target Buffer 1 (TC#-CT1), part # 15015770
- 5 Elute Target Buffer 1 (TC#-ET1), part # 15013006
- 6 Elute Target Buffer 1 (TC#-ET1), part # 15013006
- 7 Capture Target Oligos (TC#-CTO), part # 15013000
- 8 Capture Target Oligos (TC#-CTO), part # 15013000
- 9 2N NaOH (GA#-HP3), part # 11324596
- 10 2N NaOH (GA#-HP3), part # 11324596
- 11 PCR Master Mix, Polymerase (TC#-PMM), part # 15017038
- 12 PCR Primer Cocktail (TC#-PPC), part # 15017039

Consumables and Equipment

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

Table 2 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, 0.8ml (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 3 User-Supplied Equipment

Equipment	Supplier
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
2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
Magnetic stand-96	Ambion, part # AM10027
Microcentrifuge	General lab supplier
Plate centrifuge	General lab supplier
Vacuum concentrator	General lab supplier
Vortexer	General lab supplier

TruSeq DNA Sample Prep

Prior to TruSeq Exome Enrichment, you must first prepare a human DNA library using the TruSeq DNA Sample Prep kit. However, in order to support TruSeq Exome Enrichment, you must excise a band from the gel spanning the width of the lane and ranging in size from 300–400 bp, rather than 400–500 bp required for TruSeq DNA Sample Preparation. Reference the Purify Ligation Products procedures in the Illumina *TruSeq DNA Sample Preparation Guide*.

After completing the Validate Library step in TruSeq DNA Sample Preparation protocol, proceed to *First Hybridization* on page 17 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

- ▶ Capture Target Oligos (CTO)
- ▶ Capture Target Buffer 1 (CT1)

User-Supplied Consumables

- ▶ 300 μ l 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 (300–400 bp gel size fraction)



NOTE

When performing TruSeq DNA Sample Preparation for TruSeq Exome Enrichment, the required gel size fraction is 300–400 bp, smaller than the standard 400–500 bp size.

Preparation

- ▶ Remove the DNA library and 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 $^{\circ}\text{C}$ for 10 minutes
 - b 18 cycles of 93 $^{\circ}\text{C}$ for 1 minute, decreasing 2 $^{\circ}\text{C}$ per cycle
 - c 58 $^{\circ}\text{C}$ for forever

Procedure

- 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 qPCR. If pooling libraries, combine 500 ng of each DNA library. If the total volume is greater than 40 ul, use a vacuum concentrator without heat to reduce the pooled sample volume to 40 ul.

Table 4 DNA Libraries for Enrichment

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

- 2 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.

- In the order listed below, prepare the reaction mix in each well of a new 300 μ l 96-well PCR plate. Gently pipette the entire volume up and down 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 (μ l)
Capture Target Oligos	10
Capture Target Buffer 1	50
Diluted DNA library	40
Total Volume per Sample	100

- Seal the PCR 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.
- Centrifuge the PCR plate to 280 \times g for 1 minute.
- Incubate the PCR plate on the pre-programmed thermal cycler as follows:
 - 95°C for 10 minutes
 - 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle
 - 58°C for 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)

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.
- ▶ Label a new 300 µl 96-well PCR Plate "First Elution".
- ▶ [Optional] Label one new PCR tube per sample "First Elution for qPCR".

Make SMB Binding Plate

- 1 Remove the first hybridization 300 µl 96-well PCR plate from the thermal cycler.
- 2 Centrifuge the first hybridization plate to 280 xg for 1 minute.

- 3 Place the first hybridization 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 first hybridization plate to the corresponding well of a new 96-well MIDI plate using a single channel or multichannel pipette.

Binding

- 1 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 μ l of well-mixed Streptavidin Magnetic Beads to the wells of the MIDI plate containing the reaction mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 2 Seal the MIDI plate with a Microseal 'B' adhesive seal.
- 3 Let the MIDI plate stand at room temperature for 30 minutes.
- 4 Centrifuge the MIDI plate to 280 xg for 1 minute.
- 5 Remove the adhesive seal from the MIDI plate.
- 6 Place the MIDI plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.
- 7 Remove and discard all of the supernatant from each well with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 8 Remove the MIDI plate from the magnetic stand.

WS1 Clean Up

- 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 μ l Wash Solution 1 to each well of the MIDI plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to ensure the beads are fully resuspended.
- 3 Place the MIDI 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 using a single channel or multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Remove the MIDI 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 μ l Wash Solution 2 to each well of the MIDI plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 3 Place the MIDI 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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Remove the MIDI plate from the magnetic stand.
- 6 Add 200 μ l Wash Solution 2 to each well of the MIDI plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 7 Transfer the entire contents of each well of the MIDI plate to the corresponding well of a new 300 μ l 96-well PCR plate using a single channel or multichannel pipette.
- 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.



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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 14 Remove the PCR plate from the magnetic stand.
- 15 Add 200 μ l Wash Solution 2 to each sample well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10-20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 16 Repeat steps 8–13 once.

WS3 Clean Up

- 1 Remove the PCR plate from the magnetic stand.
- 2 Add 200 μ l Wash Solution 3 to each well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.
- 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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Repeat steps 1–4 once.
- 6 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.
- 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 all of the supernatant from each well with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.

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 using a single channel or multichannel pipette.
- 4 Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Ensure that the beads are fully resuspended.
- 5 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.
- 6 Let the PCR plate stand at room temperature for 5 minutes.
- 7 Centrifuge the PCR plate to 280 xg for 1 minute.
- 8 Place the PCR plate on the magnetic stand for 2 minutes until the liquid appears clear.
- 9 Carefully remove the adhesive seal from the PCR plate to avoid spilling the contents of the wells.
- 10 Transfer 29 μl of supernatant from each well of the PCR plate to the corresponding well of the new 96-well PCR plate labeled "First Elution" using a single channel or multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 11 Add 5 μl Elute Target Buffer 2 to each well of the PCR plate containing samples to neutralize the elution. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.
- 12 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.

- 13 [Optional] Dilute 2 μ l of the first elution in 98 μ l 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.

- 14 Store the remaining reagents as follows:
- Place the Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes in 2° to 8°C storage.
 - Place the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 tubes in -15° to -25°C storage.
 - 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 PCR 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

- ▶ Capture Target Oligos (CTO)
- ▶ Capture Target Buffer 1 (CT1)

User-Supplied Consumables

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

Preparation

- ▶ 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.
- ▶ 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 $^{\circ}\text{C}$ for 10 minutes
 - b 18 cycles of 93 $^{\circ}\text{C}$ for 1 minute, decreasing 2 $^{\circ}\text{C}$ per cycle
 - c 58 $^{\circ}\text{C}$ for forever

Procedure

- 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.

- In the order listed below, add the following to each well of a new 300 μ l 96-well PCR plate. 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 (μ l)
Capture Target Buffer 1	50
Capture Target Oligos	10
PCR Grade Water	10
First Elution	30
Total Volume per Sample	100

- 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.
- Centrifuge the PCR plate to 280 \times g for 1 minute.
- Incubate the PCR plate on the pre-programmed thermal cycler as follows:
 - 95°C for 10 minutes
 - 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle
 - 58°C for 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 20.

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)

User-Supplied Consumables

- ▶ 96-well MIDI Plate
- ▶ 300 μ l 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.
- ▶ Label a new 300 μ l 96-well PCR Plate "Second Elution".
- ▶ [Optional] Label one new PCR tube per sample "Second Elution for qPCR".

Make SMB Binding Plate

- 1 Remove the second hybridization 300 μ l 96-well PCR plate from the thermal cycler.
- 2 Centrifuge the room temperature second hybridization plate to 280 xg for 1 minute.
- 3 Place the second hybridization 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 second hybridization plate to the corresponding well of a new 96-well MIDI plate using a single channel or multichannel pipette.

Binding

- 1 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 μ l of well-mixed Streptavidin Magnetic Beads to the wells of the MIDI plate containing the reaction mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 2 Seal the MIDI plate with a Microseal 'B' adhesive seal.
- 3 Let the MIDI plate stand at room temperature for 30 minutes.
- 4 Centrifuge the MIDI plate to 280 xg for 1 minute.
- 5 Remove the adhesive seal from the MIDI plate.
- 6 Place the MIDI plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.
- 7 Remove and discard all of the supernatant from each well with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 8 Remove the MIDI plate from the magnetic stand.

WS1 Clean Up

- 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 μ l Wash Solution 1 to each well of the MIDI plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to ensure the beads are fully resuspended.
- 3 Place the MIDI 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 using a single channel or multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Remove the MIDI 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 μ l Wash Solution 2 to each well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 3 Place the MIDI 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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Remove the MIDI plate from the magnetic stand.
- 6 Add 200 μ l Wash Solution 2 to each well of the MIDI plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 7 Transfer the entire contents of each well of the MIDI plate to the corresponding well of a new 300 μ l 96-well PCR plate using a single channel or multichannel pipette.
- 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.



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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 14 Remove the PCR plate from the magnetic stand.
- 15 Add 200 μ l Wash Solution 2 to each sample well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly and avoid excessive bubbling or foaming. Ensure that the beads are fully resuspended.
- 16 Repeat steps 8–13 once.

WS3 Clean Up

- 1 Remove the PCR plate from the magnetic stand.
- 2 Add 200 μ l Wash Solution 3 to each well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.
- 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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 5 Repeat steps 1–4 once.
- 6 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.
- 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 with a multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.

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 using a single channel or multichannel pipette.
- 4 Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly. Ensure that the beads are fully resuspended.
- 5 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.
- 6 Let the PCR plate stand at room temperature for 5 minutes.
- 7 Centrifuge the PCR plate to 280 xg for 1 minute.
- 8 Place the PCR plate on the magnetic stand for 2 minutes until the liquid appears clear.
- 9 Carefully remove the adhesive seal from the PCR plate to avoid spilling the contents of the wells.
- 10 Transfer 29 μl of supernatant from each well of the PCR plate to the corresponding well of the new 96-well PCR plate labeled "Second Elution" using a single channel or multichannel pipette. Take care not to disturb the Streptavidin Magnetic Beads.
- 11 Add 5 μl Elute Target Buffer 2 to each well of the PCR plate containing samples to neutralize the elution. Gently pipette the entire volume of each well up and down 10-20 times to mix thoroughly.
- 12 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.

- 13 [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.

- 14 Store the remaining reagents as follows:
- Place the Streptavidin Magnetic Beads, Elute Target Buffer 2, Wash Solution 1, and Wash Solution 3 tubes in 2° to 8°C storage.
 - Place the Elute Target Buffer 1, 2N NaOH, and Wash Solution 2 tubes in -15° to -25°C storage.
 - 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)

User-Supplied Consumables

- ▶ 300 μ l 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)
- ▶ PCR Grade Water

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 8.
- ▶ 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

Make PCR

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

Reagent	Volume (µl)
Second Elution	10
PCR Master Mix	25
PCR Primer Cocktail	5
PCR Grade Water	10
Total Volume	50

- 2 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.
- 3 Centrifuge the plate to 280 xg for 5 seconds to mix thoroughly.

Amp PCR

- 1 Amplify the PCR plate on the pre-programed thermal cycler, with the lid closed, 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

Clean Up PCR



NOTE

Before performing clean up, *AMPure XP Handling* on page 8 when working with AMPure XP Beads.

- 1 Vortex the AMPure XP Beads until the beads are well dispersed, then add 90 μ l of the mixed AMPure XP Beads to each well of the PCR plate containing 50 μ l of the PCR amplified library using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 2 Incubate the PCR plate for 15 minutes at room temperature.
- 3 Place the PCR plate on the magnetic stand for 5 minutes at room temperature or until the liquid appears clear.
- 4 Using a 200 μ l single or multichannel pipette, remove and discard 140 μ l of the supernatant from each well of the PCR plate. Take care not to disturb the beads.



NOTE

Leave the PCR plate on the magnetic stand while performing the following 80% EtOH wash steps (5–7).

- 5 With the PCR plate remaining on the magnetic stand, add 200 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 6 Incubate the PCR plate for at least 30 seconds at room temperature, then remove and discard the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads.

- 7 Repeat steps 5–6 once for a total of two 80% EtOH washes.
- 8 Remove the PCR plate from the magnetic stand and let the plate stand for 15 minutes at room temperature to dry.
- 9 Resuspend the dried pellet in the each well with 30 μ l Resuspension Buffer using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 10 Incubate the PCR plate for 2 minutes at room temperature.
- 11 Place the PCR plate on the magnetic stand for 5 minutes at room temperature until the liquid appears clear.
- 12 Transfer 30 μ l of the clear supernatant from each well of the PCR plate to the corresponding well of a new 300 μ l 96-well PCR plate using a single channel or multichannel pipette.



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 PCR 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 *qPCR Quantification Protocol 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.

Data Assessment

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



NOTE

The TruSeq script can be downloaded via <http://www.illumina.com>.

Script Installation

The *install.sh* script installs the TruSeq scripts. It performs the following operations:

- 1 Copy the .pl and .pm files into the destination directory
- 2 Replace some constants in the .pl and .pm files with paths, so that the scripts can find the packages they depend on.

Syntax: `./install.sh <casava_installation_dir> <destination_dir>`

Example: `./install.sh /opt/GOAT/CASAVA_1.7.0 /home/johndoe/truseq`



NOTE

The scripts are compatible with CASAVA 1.7.0.

Script Usage

The TruSeqAnalysis.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/CPU's are supported. If you configure N TruSeq runs on the command line (with multiple `--lanes` options), the Makefile can take advantage of up to $2*N$ CPU's or cores.

There are three steps to the analysis:

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

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.



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 TruSeqAnalyses.pl script. For example, `--jobsLimit=8`.

The following is an example of how to use the TruSeq quality assessment scripts.

```
cd ~/mydata/FC123/GeraldResults
perl ~/myscripts/TruSeq1.0/TruSeqAnalysis.pl \
  --truseqRunId=FC123 \
  --lanes=1,2 \
  --lanes=3,4 \
  --targetsDir ~/myscripts/TruSeq1.0/ \
  --targetsName TruSeq_exome \
  --refSequences=/iGenomes/hg19/Sequences/chromosomes \
  --sgeAuto \
  --sgeQueue=my.q \
  --jobsLimit=8
```

Additional commonly used options include:

- e, --exportDir–Source directory PATH also known as GERALD directory or run directory (default: current directory)
- truseqProjectDir–Directory for results (default: <exportDir>/TrueSeq)
- casavaProjectDir–Directory for CASAVA build (pre-existing build will be used) (default: <exportDir>/casava_build)

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

Usage: TruSeqAnalysis.pl [options]

- targetsName–Name of targeted regions (e.g., TruSeq_exome)
- targetsDir–Directory where the targeted regions file (<targetsName>_targeted_regions.txt) is located

--truseqProjectDir–Directory for results (default: <exportDir>/TrueSeq)
--casavaProjectDir–Directory for CASAVA build (pre-existing build will be used) (default: <exportDir>/casava_build)
--truseqRunId–Run ID–Results for lane/truSeqRunId combo will be in <truseqProjectDir>/truseqRunId directory (required)
-l, --lanes–Comma-separated list of the lanes to use. Lanes will be combined. (required)
-e, --exportDir–Source directory PATH also known as GERALD directory or run directory (default: current directory)
-ref, --refSequences–PATH of the reference genome sequences (required)
--emailList–Space-separated list of email addresses.
-wa, --workflowAuto–Used for the CASAVA run. See the *CASAVA Software User Guide*.
-sa, --sgeAuto–Used for the CASAVA run. See the *CASAVA Software User Guide*.
-jobsLimit–Used for the CASAVA run. See the *CASAVA Software User Guide*.
--version–Version
--help–Prints usage guide



NOTE

- --lanes and --truseqRunId can be specified multiple times (see the Configuring Multiple Runs section of the *CASAVA Software User Guide*).
- Most of the standard CASAVA parameters can also be specified on the command line. They will be used for the CASAVA build, if it does not already exist in <casavaProjectDir>
- This script generates a Makefile in <truseqProjectDir>. It can be run with make or submitted to the SGE with qmake. Each TruSeq run (determined by the number of times --lanes is specified) will generate a CASAVA build. The number of processors used in the 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 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 $6 \cdot 100 / 300 = 2X$.

For paired end runs, the CASAVA build is run with `--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.

The directory structure of the output is as follows:

```
<truseqProjectId>
  <truseqRunId1>
  •
  •
  •
  <truseqRunIdN>
```

Each of the `<truseqRunIdX>` folders contains the output from one TruSeq analysis. If multiple different `--lanes` options are specified on the command line and a single `--truseqRunID`, then these folder names are constructed as `<truseqRunID>_<lanes>`. Alternatively, if different `--truseqRunIDs` are specified for each `--lanes` argument on the command line, then folders shown above will be named with the specified `truseqRunId`.

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

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

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

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

The results of post-alignment analysis are in the top level of the <truseqRunIdX> 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 <truseqRunIdX> folder. This is the default location for the CASAVA build, but it can be overridden on the command line.

Enrichment Statistics

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

Run ID–TrueSeqRunId, 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

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 given pool of probes.

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

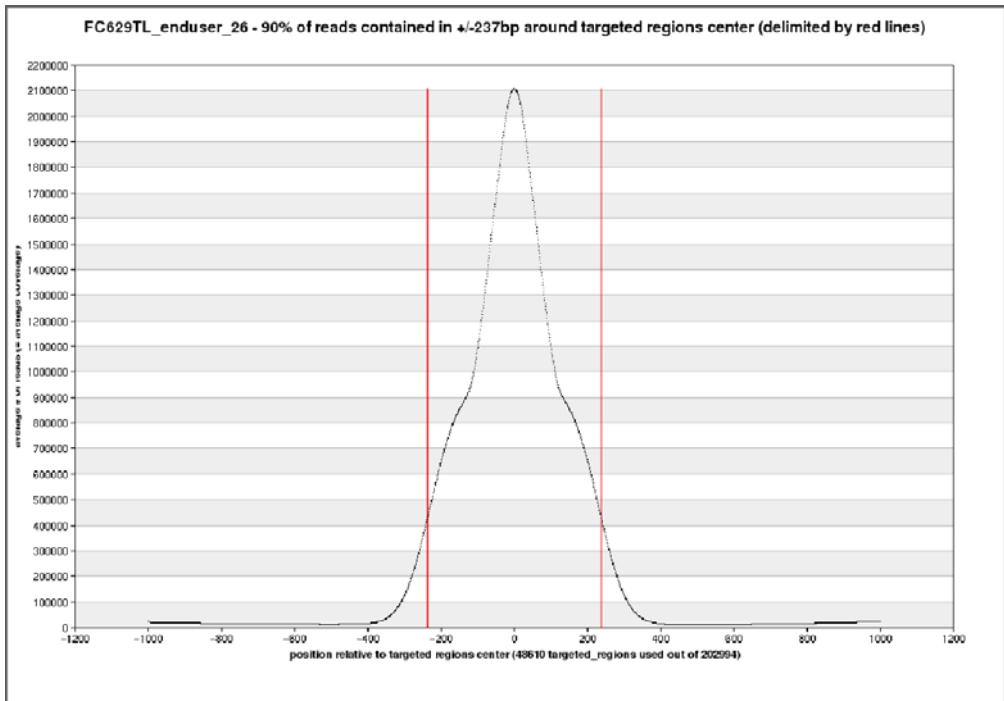
Percentage of Targeted Regions with No Reads–Percentage of targeted regions that contain 0 reads.

Read Distribution

The `<truseqRunId>_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 4 Read Distribution Plot

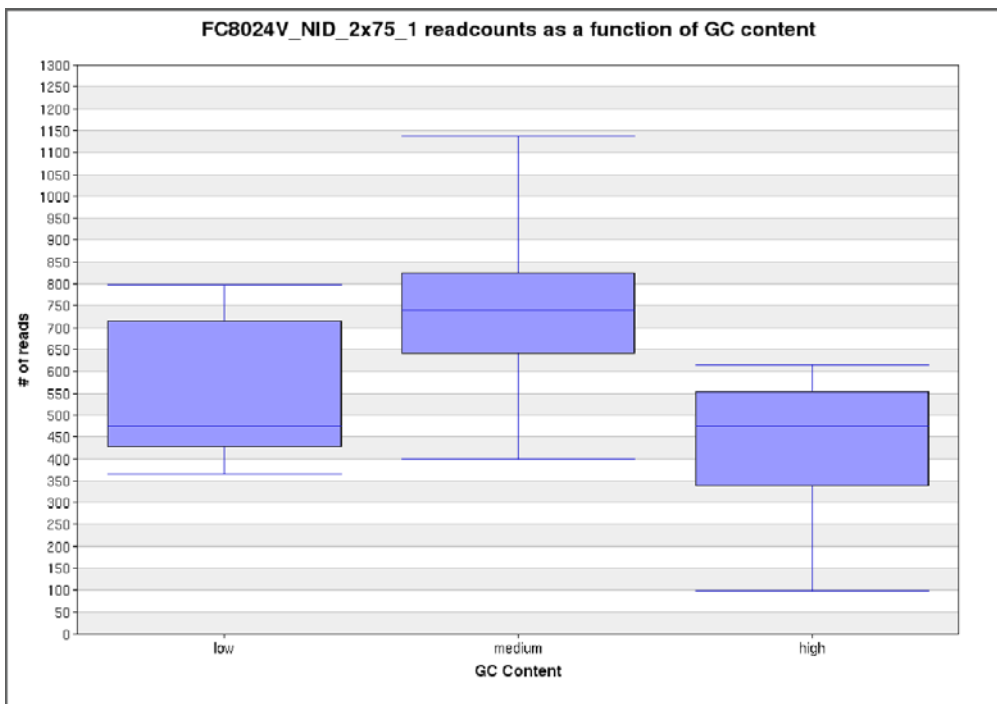


GC Control Probes

The `<truseqRunId>_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 exome.

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 `<truseqRunId>_gc_controls_readcounts.txt` file.

Figure 5 GC Control Probes Plot

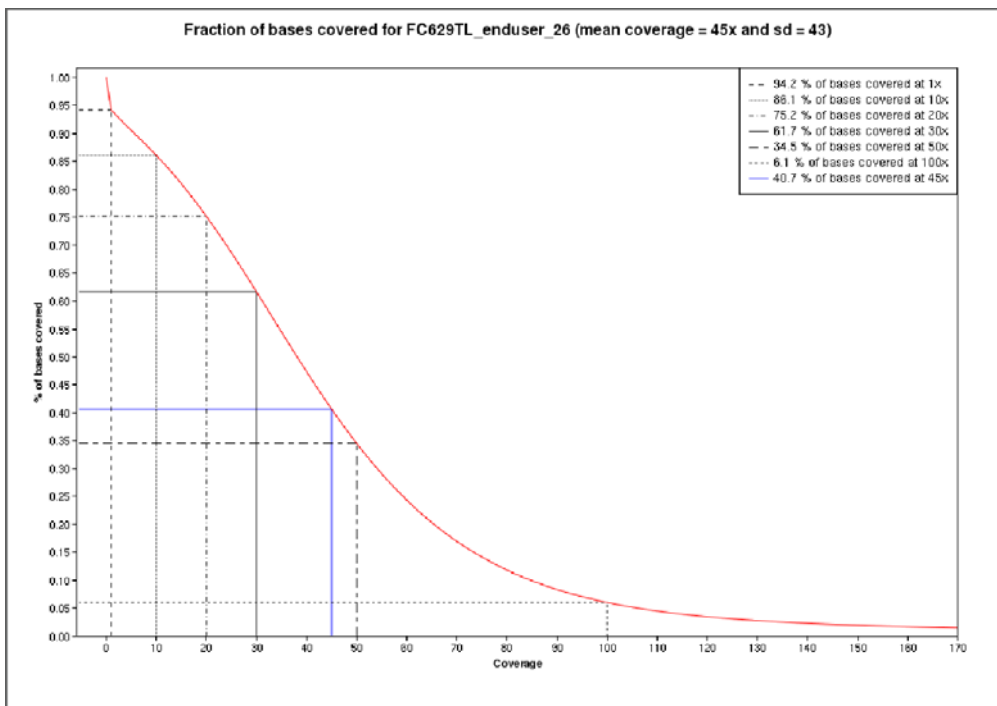


Each read that overlaps by at least one base with the probe region is counted. Duplicates are not removed. For each stringency level, the readcounts for every probe at that stringency level is used to generate the box and whiskers. The box and whisker is generated based on min, 25th percentile, median,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

Coverage Level

In the `<truseqRunId>_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 `<truseqRunId>_coverage.txt` file.

Figure 6 Coverage Level Plot

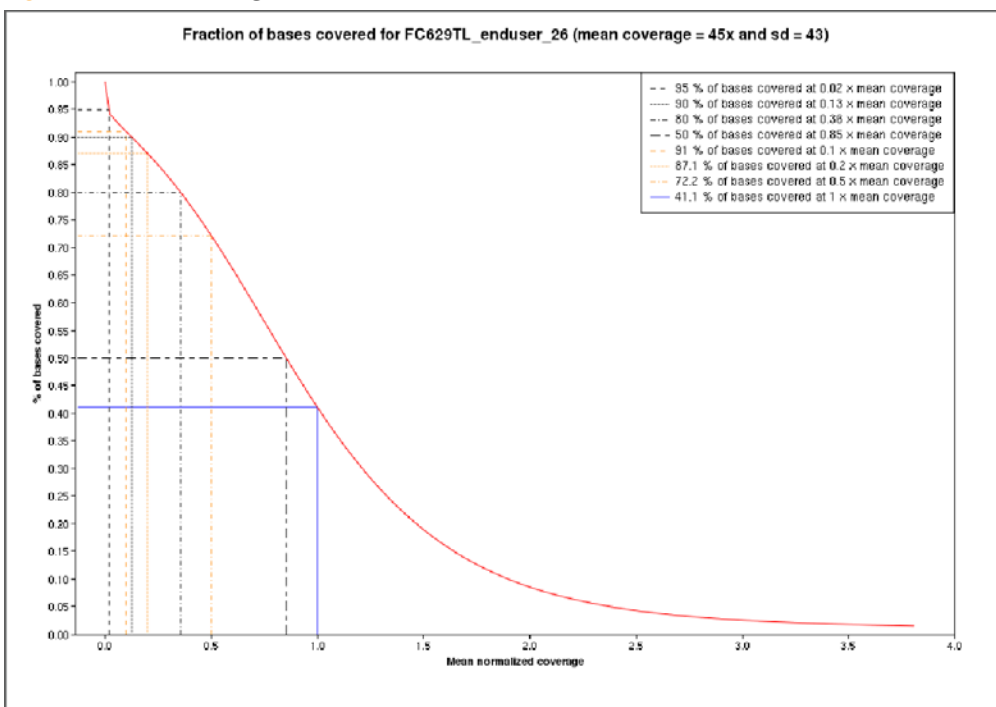


The coverage level at every base in the targeted regions is extracted from the CASAVA build. Statistics are then calculated on this data set: mean, standard deviation, and percentage of bases at or above a given coverage level.

Mean Coverage Level

The `<truseqRunId>_Fraction_bases_covered_mean_normalized.png` file is similar to the `<truseqRunId>_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 7 Mean Coverage Level Plot

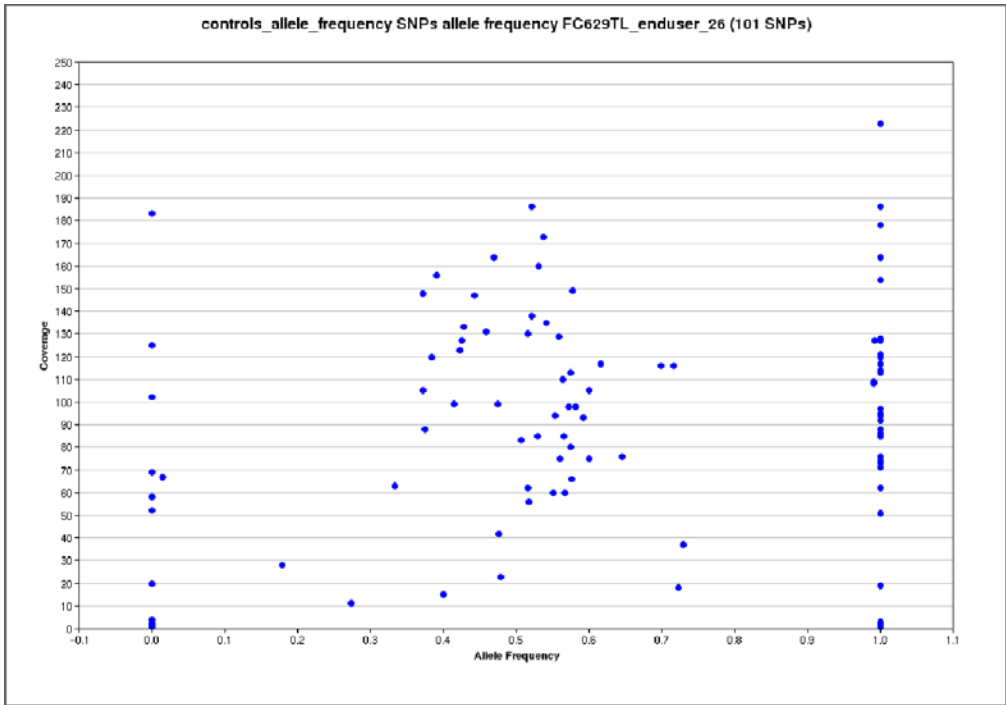


As an example, if two runs have a substantially different number of total reads, the percentage of bases that are covered at 0.2 times the mean coverage level can be compared to better understand whether the coverage levels would be comparable if there were the same total number of reads for both samples.

SNP Control Probes

The `<truseqRunId>_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 8 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.

Coverage

This `<truseqRunId>_coverage.txt` file contains four tables:

- ▶ The first table contains the same data shown in the legend of the plot `<truseqRunId>_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 (50%, 80%, 90%, 95%). 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 coverage.

Controls

This section describes the sample-independent and sample-dependent controls used in TruSeq Exome 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 CTO pool to target these dsDNA controls. Although Illumina recommends selecting the 300–400 bp gel fraction to generate the input library for enrichment, 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 1.8 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 5–15 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. 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 (see the *GC Control Probes Plot* on page 45). 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 exome 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.

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 (see the *SNP Control Probes* on page 48). 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 exome enrichment.

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Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 5 Illumina General Contact Information

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

Table 6 Illumina Customer Support Telephone Numbers

Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850
Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

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>.

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