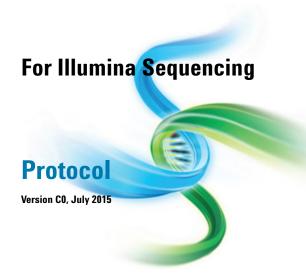


HaloPlex Exome Target Enrichment System



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In this Guide...

This guide describes an optimized protocol for using the HaloPlex Exome target enrichment system to prepare sequencing library samples for Illumina paired-end multiplexed sequencing platforms.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps of the HaloPlex workflow, to prepare exome target-enriched sequencing libraries for the Illumina platform.

3 Reference

This chapter contains reference information, including component kit contents, index sequences, and optional gel validation instructions.

What's New in Version C.0

• Updated product labeling statement.

What's New in Version B.0

 Support for kits supplied with either of two indexing primer configurations.

Kits with revised index configuration (typically received December, 2014 or later) include indexing primers A01 through H12 provided in white-capped tubes (16 Reaction kits) or in a blue plate (96 Reaction kits). For kit content details see page 52. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 13 on page 55.

Kits with original index configuration (typically received before December, 2014), include indexing primers 1–96 provided in clear-capped tubes (16 Reaction kits) or in a clear plate (96 Reaction kits). For kit content details see page 56. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 17 on page 59 through Table 22 on page 64.

Protocol steps for indexing using primers provided in either configuration are identical.

- Hybridization duration updated to range of 16 to 24 hours (Table 5 on page 31)
- Updated ordering information for Agilent 2200 TapeStation consumables (Table 3 on page 11)
- Updated supplier information for NaOH and acetic acid (Table 1 on page 9)
- Support for FFPE-derived DNA samples (see Note on page 16)
- Instructions for obtaining Agilent's SureCall analysis software (page 49)
- Updates to illustrations of the HaloPlex exome target-enrichment workflow (Figure 1 on page 14) and expected products (Figure 7 on page 41)

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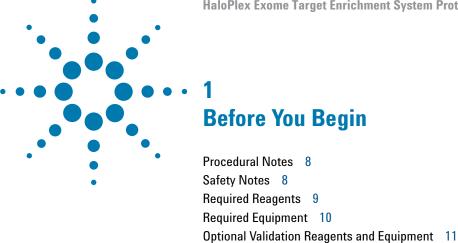
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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Procedural Notes

- The protocols in this manual are for use with Agilent's G9906A and G9906B HaloPlex Exome Target Enrichment System kits. The protocol is not compatible with HaloPlex reagent kits supplied with custom HaloPlex probe designs.
- The HaloPlex protocol is optimized for digestion of 200 ng of genomic DNA (split among 8 different restriction digestion reactions) plus 25 ng excess DNA, for a total of 225 ng genomic DNA. Using lower amounts of DNA in the enrichment protocol can adversely affect your results. Use a fluorometry-based DNA quantitation method, such as PicoGreen stain or Qubit fluorometry to quantify the DNA starting material.
- Always keep pre-amplification and post-amplification DNA samples in separate work areas. Perform the enrichment procedure in the pre-amplification area. Open and store the amplified, enriched DNA samples only in the post-amplification area.
- Possible stopping points, where DNA samples may be stored between steps, are marked in the protocol. Store the samples at -20°C, but do not subject the samples to multiple freeze/thaw cycles.
- Ensure that master mixes are thoroughly mixed, by pipetting up-and-down or by gentle vortexing, before distributing to the samples.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes



Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

 Table 1
 Required Reagents for HaloPlex Target Enrichment

Description	Vendor and part number
HaloPlex Exome Target Enrichment System Kit (select one)	Agilent
ILM, 16 reactions	p/n G9906A
ILM, 96 reactions*	p/n G9906B
Herculase II Fusion Enzyme with dNTPs (100 mM; 25 mM for each nucleotide), 200 reactions [†]	Agilent p/n 600677
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
10 M NaOH, molecular biology grade	Sigma, p/n 72068
2 M acetic acid	Sigma, p/n A8976
10 mM Tris-HCl, pH 8.0 or 10 mM Tris-acetate, pH 8.0	General laboratory supplier
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer	
100 assays, 2-1000 ng	Life Technologies p/n Q32850
500 assays, 2-1000 ng	Life Technologies p/n Q32853

^{*} Kits contain enough reagents for 16 or 96 reactions total, including one or more control reactions using Enrichment Control DNA (ECD) samples. Each run of up to 96 samples should include one ECD control enrichment reaction.

[†] Also available separately as Herculase II Fusion DNA Polymerase, 40 reactions (Agilent p/n 600675) and 100 mM dNTP Mix (Agilent p/n 200415, sufficient for 1000 HaloPlex enrichment reactions).

1 Before You Begin

Required Equipment

Required Equipment

 Table 2
 Required Equipment for HaloPlex Target Enrichment

Description	Vendor and part number
Thermal Cycler	Agilent SureCycler 8800, p/n G8800A and 96 well plate module, p/n G8810A or equivalent thermal cycler and accessories*
Thermal cycler-compatible 96-well plates	Agilent p/n 401333 (for SureCycler 8800) or see manufacturer's recommendations
8-well PCR strip tubes with caps	Nippon Genetics, p/n FG-088WF, or equivalent
96-well plate and strip tube-compatible magnetic separator	Agencourt SPRIPlate Super Magnet Plate p/n A32782, or equivalent
1.5 mL tube-compatible magnetic separator	DynaMag-2 magnet, Life Technologies p/n 12321D, or equivalent
Benchtop microcentrifuge	VWR p/n 93000-196, or equivalent
Benchtop plate centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000, or equivalent
Multichannel pipettes (10-μL and 100-μL volume)	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Adhesive seals for 96-well PCR plates	Agilent p/n 410186, or equivalent
Qubit 2.0 Fluorometer	Life Technologies p/n Q32866
Qubit assay tubes	Life Technologies p/n Q32856
Ice bucket	General laboratory supplier
Vortex mixer	General laboratory supplier

 $^{^{\}ast}$ Thermal cycler must have a maximum reaction volume specification of at least 100 μL and must be compatible with 0.2 mL tubes.

Optional Validation Reagents and Equipment

 Table 3
 Reagents and Equipment for Optional Validation Methods

Description	Vendor and part number	
2200 TapeStation Platform and Consumables		
2200 TapeStation	Agilent p/n G2964AA or G2965AA	
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584	
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585	
2100 Bioanalyzer Platform and Consumables		
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA	
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA	
High Sensitivity DNA Kit	Agilent p/n 5067-4626	
Gel Electrophoresis Platform and Consumables		
XCell SureLock Mini-cell	Life Technologies p/n El0001	
Novex 6% Polyacrylamide, TBE Pre-cast Gels	Life Technologies p/n EC62655BOX	
Novex TBE Running Buffer, 5X	Life Technologies p/n LC6675	
Novex High-density TBE Sample Buffer, 5X	Life Technologies p/n LC6678	
GelRed Nucleic Acid Stain, 3X in water	Biotium p/n 41001	
DNA molecular weight markers	General laboratory supplier	
UV-transilluminator	General laboratory supplier	

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This section contains instructions for gDNA library target enrichment for sequence analysis using the Illumina platform. For each sample to be sequenced, an individual target-enriched, indexed library is prepared.

The HaloPlex Target Enrichment System amplifies thousands of targets in the same reaction, incorporating standard Illumina paired-end sequencing motifs in the process. During hybridization, each sample can be uniquely indexed, allowing for pooling of multiple samples per sequencing lane.

See Figure 1 for a summary of the overall HaloPlex exome target enrichment workflow.



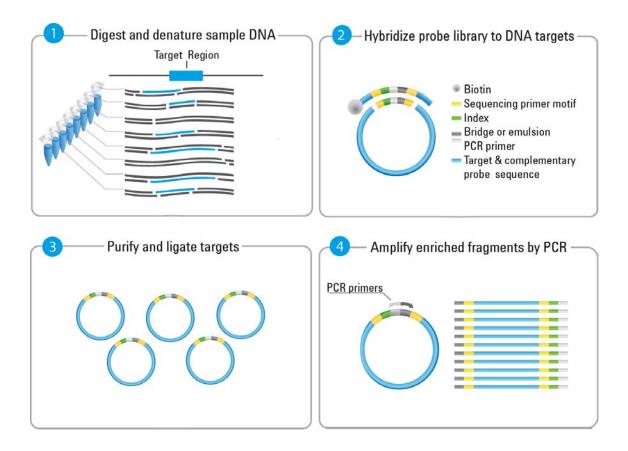


Figure 1 Overall HaloPlex exome target-enriched sequencing sample preparation workflow.

Run Size Considerations

Kits contain enough reagents for 16 or 96 reactions total, including control reactions using the provided Enrichment Control DNA (ECD). Each run that uses independently-prepared reagent master mixes should include one ECD control enrichment reaction.

The following protocol includes volumes appropriate for 16-sample runs. When planning a run size different from 16 samples, you will need to adjust volumes of components accordingly. Calculate the amount of each solution needed for the number of reactions in your run, plus 2 reactions excess for the restriction digestion steps and for preparation of the hybridization master mixes and using 1 reaction excess for the remaining steps. For example, for a 24 reaction run, calculate amounts of each solution by multiplying the single reaction value by 26 for restriction digestion and hybridization steps and by 25 for later steps.

A 16-reaction kit contains enough reagents to prepare master mixes for one run of 16 samples. When processing samples using runs with fewer than 16 samples, some reagents may be depleted before 16 samples are run.

A 96-reaction kit contains enough reagents to prepare master mixes for 6 runs of 16 samples each, for a total of 96 samples. When processing samples using runs with fewer than 16 samples, some reagents may be depleted before 96 samples are run.

Setting up a 16-sample run using 8 DNA samples on each of two 96-well plates facilitates efficient liquid handling during the protocol. For this reason, the figures provided in the protocol illustrate the restriction digestion and hybridization steps showing 8 DNA samples per 96-well plate. Later steps in the protocol are most efficiently done using 8-well strip tubes.

Step 1. Digest genomic DNA with restriction enzymes

In this step, gDNA samples are digested by 16 different restriction enzymes to create a library of gDNA restriction fragments. The gDNA is digested in eight different restriction reactions, each containing two restriction enzymes. The 16 restriction enzymes are provided in two 8-well strip tubes that are distinguished by red and green color markers. Enzymes are combined from corresponding wells of the red- and green-marked strip tubes to make eight different RE Master Mixes, which are then combined with each DNA sample in the run.

NOTE

Successful enrichment requires high-quality and carefully quantified DNA samples.

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Verify the size distribution of DNA in each DNA preparation by gel electrophoresis. Any smearing below 2.5 kb indicates sample degradation.

For HaloPlex target enrichment of FFPE-derived DNA samples, see Agilent publication no. G9900-90050, available at http://www.genomics.agilent.com. This publication provides a PCR-based protocol for assessment of DNA integrity and provides HaloPlex protocol modifications for improved performance from lower-quality DNA samples.

In the protocol below, 200 ng genomic DNA is split among eight different restriction digests, with an additional 25 ng excess DNA included to allow for pipetting losses. Using <225 ng DNA in the enrichment protocol can result in low yield and can potentiate rare allele dropouts. Use a fluorometry-based DNA quantitation method, such as Qubit fluorometry or PicoGreen staining, to accurately quantify the DNA starting material.

- 1 Use the Qubit dsDNA BR Assay or PicoGreen staining kit to determine the concentration of your gDNA samples. Follow the manufacturers instructions for the kits and instruments.
- **2** Prepare the DNA samples for the run. For 16-reaction runs, prepare 15 gDNA samples and one Enrichment Control DNA sample.
 - a In separate tubes of 0.2-mL PCR tube strips, dilute 225 ng of each gDNA sample in 45 μ L nuclease-free water, for a final DNA concentration of 5 ng/ μ L. Store on ice.
 - **b** In one separate tube of the appropriate strip, dispense 45 μL of the supplied Enrichment Control DNA (ECD). Store on ice.

NOTE

Although specific instructions are provided for the typical 16-sample run size, runs may include up to 96 samples. Include one ECD control sample per run of 2-96 samples. See page 15 for additional run size considerations.

3 Prepare the Restriction Enzyme Master Mix strip.

NOTE

Keep Enzyme Strips 1 and 2 and the RE Master Mixes on ice until you start the restriction digest reactions on page 20.

In this step, eight separate restriction enzyme master mixes are prepared by combining two enzymes (from Enzyme Strips 1 and 2), restriction buffer, and BSA in each well of an 8-well strip tube. Figure 2 illustrates how to prepare the Restriction Enzyme Master Mix strip for a 16-sample run using the steps detailed on page 18.

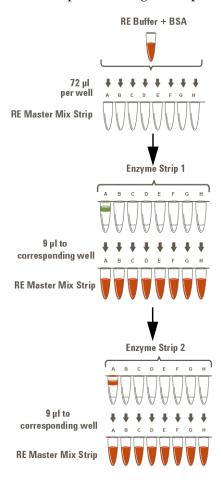


Figure 2 Preparation of the Restriction Enzyme Master Mix Strip for 16-sample run.

Step 1. Digest genomic DNA with restriction enzymes

For runs of greater or fewer than 16 samples, calculate volumes needed using the number of reactions in the run plus 2 reactions excess.

a Combine the amounts of RE Buffer and BSA Solution indicated in the table below in a 1.5-mL tube. Mix by vortexing briefly.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
RE Buffer	34.0 μL	612 µL
BSA Solution	0.85 μL	15.3 μL
Total Volume	34.85 μL	627.3 μL

b To begin preparation of the Restriction Enzyme Master Mix Strip, dispense the appropriate volume of the RE Buffer/BSA mixture to each well of a fresh 8-well strip tube.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
RE Buffer/BSA	4 μL	72 μL

CAUTION

It is important to use the restriction enzyme tube strip in the proper orientation when preparing the RE Master Mixes as described below. The red or green color marker on the tube strip and cap strip is positioned adjacent to well A of each enzyme strip.

c Using a multichannel pipette, add the appropriate volume of each enzyme from Enzyme Strip 1 (green marker on tube A) to each corresponding tube A to H of the Restriction Enzyme Master Mix Strip. Keep the RE Master Mix Strip on ice.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
RE Enzymes from Enzyme Strip 1	0.5 μL	9 μL

d Using a multichannel pipette, add the appropriate volume of each enzyme from Enzyme Strip 2 (with red marker on tube A) to each corresponding tube A to H of the same Restriction Enzyme Master Mix Strip. Keep the RE Master Mix Strip on ice.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
RE Enzymes from Enzyme Strip 2	0.5 μL	9 μL

- **e** Mix by gentle vortexing and then spin briefly.
- **f** Keep the Restriction Enzyme Master Mix Strip on ice until it is used in step 4.
- **4** Aliquot the Restriction Enzyme Master Mixes to the rows 96-well plates to be used as the restriction digest reaction plates.

NOTE

Multichanel pipetting in later protocol steps is facilitated by configuring a 16-sample run as 8 DNA samples in columns 1-8 on each of two 96-well plates. The figures in the protocol below illustrate liquid handling steps for one of the plates with 8 DNA samples.

- **a** Align the Restriction Enzyme Master Mix Strip, prepared in step 3, along the vertical side of a 96-well PCR plate as shown below.
- **b** Using a multichannel pipette, carefully distribute 5 μ L of each RE master mix row-wise into each well of columns 1-8. For 16-sample runs, continue pipetting 5 μ L of each RE master mix row-wise into columns 1-8 of a second 96-well plate.

For runs with >16 samples, continue distributing 5 μ L from the same RE Master Mix strip row-wise into additional 96-well plates.

Visually inspect pipette tips for equal volumes before dispensing to the plate(s).

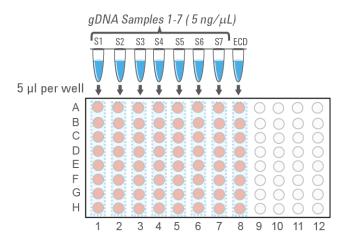
Α RE Master Mix Strip В С D Е G G Н 2 3 5 1 4 6 7 8 9 10 12

Restriction Digestion Reaction Plate

Each row of the 96-well plates now contains 5 μL per well of the same restriction enzyme combination.

Step 1. Digest genomic DNA with restriction enzymes

- **5** Aliquot DNA samples into the 96-well Restriction Digest Reaction Plates.
 - **a** Align the DNA samples prepared in step 2 (15 gDNA samples and the ECD sample for a 16 sample run) along the horizontal side of the digestion reaction plates as shown below.



- \boldsymbol{b} Carefully distribute 5 μL of DNA samples column-wise into each well of the digestion reaction plate.
 - If using a multichannel pipette, visually inspect pipette tips for equal volumes before dispensing.
- **c** Seal the plates thoroughly with adhesive plastic film.
- **6** Carefully vortex the plates to mix the digestion reactions.
- **7** Briefly spin the plates in a plate centrifuge.

Wells of the prepared 96-well plates now contain complete 10- μL restriction digestion reactions. In this format, each column corresponds to one DNA sample digested in eight different restriction reactions.

8 Place each Restriction Digest Reaction Plate in a thermal cycler and run the program in Table 4, using a heated lid.

 Table 4
 Thermal cycler program for HaloPlex restriction digestion

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	8°C	Hold

NOTE

Do **not** pool the eight restriction digests for a single DNA sample. For target selection using the HaloPlex exome probe library, the eight restriction digests for each DNA sample are hybridized separately to a different collection of probe molecules. Enriched samples are pooled **after** the hybridization step on page 32.

9 Validate the restriction digestion reaction by electrophoretic analysis of the Enrichment Control DNA (ECD) reactions.

Keep the Restriction Digest Reaction Plate on ice during validation.

- a Transfer 4 μL of each ECD digestion reaction from wells of the digestion reaction plate to fresh 0.2-mL PCR tubes.
- **b** Add back 4 μ L of nuclease-free water to each of the ECD digestion reaction wells to compensate for the volume of sample removed in step a, in order to allow processing of ECD samples in later workflow steps.
- c Incubate the removed 4-µL samples at 80°C for 5 minutes to inactivate the restriction enzymes.
- **d** Analyze the prepared samples using microfluidic electrophoresis on the 2100 Bioanalyzer (see page 23) or on the 2200 TapeStation (see page 24) or by gel electrophoresis (see page 25).

The ECD sample contains genomic DNA mixed with an 800-bp PCR product that contains restriction sites for all the enzymes used in the digestion protocol. When analyzing validation results, the undigested control should have gDNA bands at >2.5 kbp and a PCR product band at 800 bp. Each of the eight digested ECD samples should have a smear of gDNA restriction fragments between 100 and 2500 bp, overlaid with three predominant bands at approximately 125, 225 and 450 bp. These

Step 1. Digest genomic DNA with restriction enzymes

three bands correspond to the 800-bp PCR product-derived restriction fragments, and precise sizes will differ after digestion in each of the eight RE master mixes.

NOTE

In addition to the three predominant bands at approximately 125, 225 and 450 bp, you may detect additional, minor bands in the digested ECD sample lanes.

Successful digestion is indicated by the appearance of the three predominant bands. The presence of additional minor bands, with relative abundance similar to the additional bands visible in Figure 3, Figure 4, and Figure 5 does not impact enrichment results.

It is acceptable for band intensities in digestion reaction B to be slighltly reduced, compared to the other digestion reactions.

Option 1: Validation by 2100 Bioanalyzer analysis

Use a High Sensitivity DNA Kit (p/n 5067-4626) and the 2100 Bioanalyzer system with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer system setup instructions.

- Prepare an undigested DNA gel control by combining 0.5 μL of the Enrichment Control DNA stock solution and 3.5 μL of nuclease-free water.
- Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μL of each ECD sample and undigested DNA control for the analysis.
- When loading samples on the chip, load the DNA ladder in the ladder sample well marked on the chip. Load the eight ECD digest samples (A to H) in sample wells 1 to 8, and load the undigested ECD sample in sample well 9. Do not run the undigested ECD control in sample well 1.
- Place the prepared chip into the 2100 Bioanalyzer instrument and start the run within five minutes after preparation.

See Figure 3 for sample Bioanalyzer electrophoresis results.

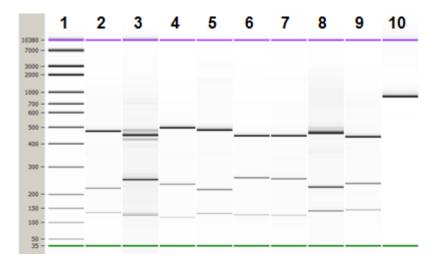


Figure 3 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

Step 1. Digest genomic DNA with restriction enzymes

Option 2: Validation by 2200 TapeStation analysis

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585). For more information to do this step, see the 2200 TapeStation User Manual.

- Prepare an undigested DNA gel control by combining 1 μL of the Enrichment Control DNA solution and 1 μL of nuclease-free water.
- Prepare the TapeStation samples as instructed in the 2200
 TapeStation User Manual. Use 2 μL of each ECD sample diluted with 2 μL of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.

CAUTION

Make sure that you thoroughly mix the combined DNA and High Sensitivity D1000 sample buffer on a vortex mixer for 5 seconds for accurate results.

• Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the 2200 TapeStation User Manual. Start the run.

See Figure 4 for sample TapeStation electrophoresis results

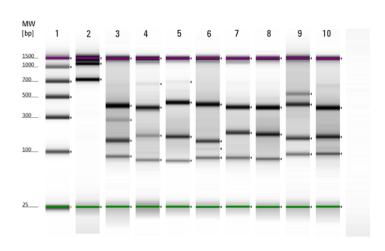


Figure 4 Validation of restriction digestion by 2200 TapeStation analysis. Lane 1: TapeStation D1000 High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

Option 3: Validation by gel electrophoresis

Use a Novex 6% polyacrylamide TBE pre-cast gel and 1X Novex TBE Running Buffer. For more information to do this step, consult the manufacturer's recommendations.

- Prepare an undigested DNA gel control by combining 2 μL of the Enrichment Control DNA stock solution and 2 μL of nuclease-free water.
- Add 1 μL of Novex Hi-Density TBE Sample Buffer (5X) to each 4- μL ECD sample.
- Load 5 μL of each sample on the gel. In one or more adjacent lanes, load 200 ng of a 50-bp DNA ladder.
- Run the gel at 210 V for approximately 15 minutes.
- Stain the gel in 3X GelRed Nucleic Acid Stain for 10 minutes, and visualize bands under UV radiation.

See Figure 5 for sample gel results.

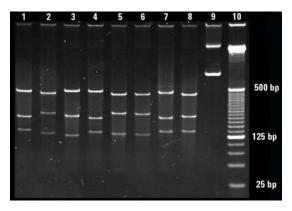


Figure 5 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

Stopping Point

If you do not continue to the next step, samples may be stored at -20°C for long-term storage. There are no more long-term stopping points until after the PCR amplification step on page 38.

Step 2. Hybridize HaloPlex probes A-H to DNA digests A-H in the presence of indexing primers

Step 2. Hybridize HaloPlex probes A-H to DNA digests A-H in the presence of indexing primers

The HaloPlex exome probe is provided as eight separate probe solutions in wells A–H of the HaloPlex Probe 8-well Strip. In this step, the eight probe solutions are hybridized, in eight separate hybridization wells, to the DNA restriction fragment libraries from Step 1. The hybridization reactions must be prepared by matching the probe tubes A-H to DNA digested using the corresponding Restriction Enzyme Master Mixes A-H.

DNA from a particular sample that is digested and then hybridized in eight separate reactions (rows A-H of a column) is combined **after** hybridization.

CAUTION

Do **not** pool the probe solutions provided in the HaloPlex Probe 8-well Strip. The eight probe pools must be used for hybridization in separate wells, with the captured DNA being combined after hybridization.

HaloPlex probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences are incorporated into the targeted fragments.

For sample indexing primer assignments, see the Reference chapter for the nucleotide sequences of the 96 indexes used in the HaloPlex Exome Target Enrichment System.

CAUTION

This guide includes information for kits containing two different sets of indexing primers. Verify that you are referencing the information appropriate for your kit version before you proceed.

Kits with indexing primers supplied in white-capped tubes or blue plate include 8-bp indexes A01 through H12. See page 52 through page 55 for indexing primer A01–H12 format and nucleotide sequence information.

Kits with indexing primers supplied in clear-capped tubes or clear plate include 8-bp indexes 1 through 96. See page 56 through page 64 for indexing primer 1–96 format and nucleotide sequence information.

Protocol steps for indexing using primers provided in either configuration are identical.

1 Prepare a Hybridization Master Mix Strip, containing Hybridization Solution and one of the eight probe solutions A-H per well. Figure 6 illustrates how to prepare the Hybridization Master Mix strip for an 16-sample run using step a to step c detailed below.

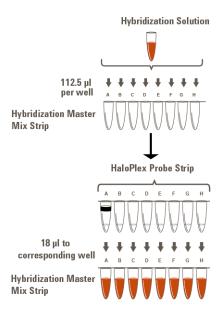


Figure 6 Preparation of the Hybridization Master Mix Strip for 16-sample run.

a Dispense the appropriate volume of Hybridization Solution to each well of a fresh 8-well strip tube. Calculate the volume needed using the number of reactions in the run plus 2 reactions excess.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
Hybridization Solution	6.25 μL	112.5 µL

Step 2. Hybridize HaloPlex probes A-H to DNA digests A-H in the presence of indexing primers

CAUTION

It is important to use the HaloPlex Probe tube strip in the proper orientation when preparing the Hybridization Master Mixes as described below. The black color marker on the tube strip and cap strip is positioned adjacent to probe well A.

b Using a multichannel pipette, add the appropriate volume of probe solution from the HaloPlex Probe Strip (with black marker near tube A) to each corresponding tube A to H of the Hybridization Master Mix Strip. Calculate the volume needed using the number of reactions in the run plus 2 reactions excess.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
HaloPlex Probes from provided strip tube	1 μL	18 μL

- **c** Mix well by gentle vortexing, then spin the tube briefly.
- 2 Prepare the Hybridization plate using the following components, using the detailed instructions in step a to step d below:
 - Restriction Digest Reaction Plate, held on ice, from page 21
 - Hybridization Master Mix Strip, prepared in step a above
 - HaloPlex Indexing Primer Cassettes (supplied in tubes or 96-well plate)

CAUTION

This guide includes information for kits containing two different sets of indexing primers. Verify that you are referencing the information appropriate for your kit version before you proceed.

Reference information for kits with indexing primers supplied in white-capped tubes or blue plate begins on page 52.

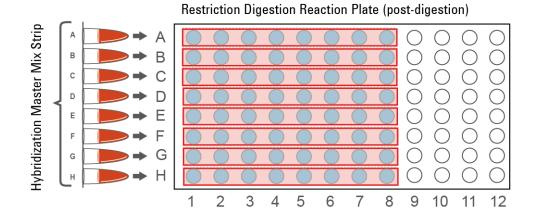
Reference information for kits with indexing primers supplied in clear-capped tubes or clear plate begins on page 56.

Protocol steps for indexing using primers provided in either configuration are identical.

a Aliquot the Hybridization Master Mixes to the rows of the digestion plate as shown below. Align the Hybridization Master Mix Strip along the vertical side of the 96-well Restriction Digest Plate. Using a multichannel pipette, carefully distribute 7.25 µL of each Hybridization Master Mix row-wise into each digested DNA sample well of the plate.

For runs using multiple plates, continue distributing 7.25 μL from the Hybridization Master Mix row-wise into the digested DNA sample wells of additional plates.

Visually inspect pipette tips for equal volumes before dispensing to the plate(s).

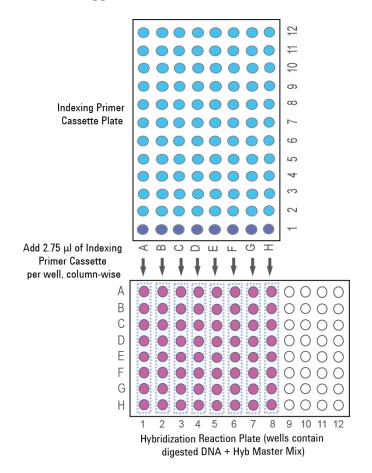


Step 2. Hybridize HaloPlex probes A-H to DNA digests A-H in the presence of indexing primers

b Add 2.75 μL of the appropriate Indexing Primer Cassette to all eight wells of the column containing the appropriate gDNA sample.

Be sure to add only one specific Indexing Primer Cassette to each column. Record the identity of the Indexing Primer Cassette added to each column for later sequence analysis.

The example in the figure below shows addition of indexes from column 1 the 96-well plate supplied with 96-reaction kits. In this situation, multichannel pipetting is facilitated by orienting the Indexing Primer Cassette plate perpendicular to the Hybridization plate. For 16-reaction HaloPlex Exome kits, Indexing Primer Cassettes are supplied in individual tubes.



- **c** Seal the plates thoroughly with adhesive plastic film to prevent evaporation.
- **d** Carefully vortex the plates to mix the hybridization reactions and then spin the plate briefly in a plate centrifuge.
- **3** Place each sealed Hybridization Plate in a thermal cycler and run the program in Table 5.

Use a heated lid. Do **not** include a low-temperature hold step in the thermal cycler program. Incubation at 54°C for more than the indicated time is not recommended.

Table 5 Thermal cycler program for HaloPlex Exome probe hybridization

Step	Temperature	Time
Step 1	95°C	10 minutes
Step 2	54°C	16–24 hours

Step 3. Pool and capture the target DNA

In this step, the circularized target DNA-HaloPlex probe hybrids, containing biotin, are captured on streptavidin beads. The eight hybridization reactions that correspond to one DNA sample are combined in this step for capture in a single capture reaction.

- 1 Remove reagents to be used in upcoming protocol steps from cold storage and allow the solutions to reach room temperature:
 - From -20°C storage, remove the Capture Solution, Wash Solution, Ligation Solution and SSC Buffer.
 - From +4°C storage, remove the HaloPlex Magnetic Beads.
- 2 Prepare 1 μL per sample, plus excess, of 2 M acetic acid for use on page 36.
- 3 Prepare 50 μ L per sample, plus excess, of fresh 50 mM NaOH for use in the DNA elution step on page 37.

Prepare the 50 mM NaOH solution from a 10 M NaOH stock solution.

CAUTION

Using high-quality NaOH is critical for optimal DNA elution and recovery.

- Do not use stock NaOH solutions that were stored at concentrations below 10 M to prepare the 50 mM NaOH solution.
- Keep the 50 mM NaOH solution container sealed when not in use, especially when processing large numbers of samples per run.
- **4** Vigorously resuspend the provided HaloPlex Magnetic Beads on a vortex mixer. The magnetic beads settle during storage.
- 5 Prepare 40 μL of HaloPlex Magnetic Beads per hybridization sample, plus excess, for the capture reaction:
 - **a** Transfer the appropriate volume of bead suspension to a 1.5-mL tube. Calculate the volume needed using the number of reactions in the run plus 1 reaction excess.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
HaloPlex Magnetic Bead suspension	40 μL	680 μL

b Put the tube into a 1.5 mL tube-compatible magnetic rack for 5 minutes.

- **c** After verifying that the solution has cleared, carefully remove and discard the supernatant using a pipette.
- **d** Add an equivalent volume of Capture Solution to the beads and resuspend by pipetting up and down.

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
Capture Solution	40 μL	680 μL

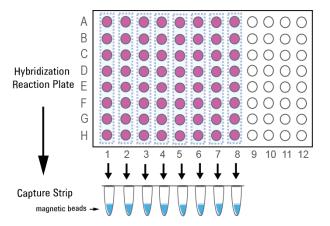
6 Using 8-well strip tubes, prepare the appropriate number of Capture Strips as described below, using one tube for each DNA sample in the run. For a 16-sample run, prepare two 8-tube Capture Strips.

To prepare each Capture Strip, pipette 40 μL of the prepared bead suspension into each tube of the 8-tube strip.

NOTE

When adding beads to the tube strip, visually inspect the bead preparation to ensure a homogeneous suspension with no aggregated bead mass at the bottom of the tube. If aggregation is present, thoroughly resuspend the beads by vortexing and pipetting up and down before use.

- **7** Remove the hybridization plates from the thermal cycler and spin the plates briefly in a plate centrifuge.
- 8 Using a multichannel pipette, transfer the full volume (approximately $20~\mu L$) of each hybridization reaction directly into the appropriate tube of the Capture Strip. Combine all eight hybridization reactions that correspond to one DNA sample (all wells of a single column) into a single capture reaction tube.



Step 3. Pool and capture the target DNA

NOTE

Due to partial evaporation of samples, you may recover less than 20 μ L of each hybridization reaction. Minor reductions in volume will not impact capture performance; you do not need to compensate for any sample evaporation volume losses in the final pooled capture reaction.

- **9** Mix the capture reactions thoroughly by pipetting up and down 15 times using a 100-μL pipette set to 80 μL.
- **10** Incubate the capture reactions at room temperature for 15 minutes.
- 11 Briefly spin the Capture Strips in a desktop centrifuge and then transfer the strips to the Agencourt SPRIPlate Super Magnet magnetic plate.

NOTE

Use the Agencourt SPRIPlate Super Magnet magnetic plate for the remainder of magnetic bead collection steps.

- **12** Wait for the solution to clear (about 30 seconds), then remove and discard the supernatant using a pipette set to 200 μL.
- **13** Wash the bead-bound samples:
 - a Remove the capture reaction tubes from the magnetic plate and add $100~\mu L$ of Wash Solution to each tube.
 - **b** Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-μL multichannel pipette set to 80 μL.
 - **c** Incubate the tubes in a thermal cycler at 46°C for 10 minutes, using a heated lid.
 - Do **not** include a low-temperature hold step in the thermal cycler program following the 10-minute incubation.
 - **d** Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic plate.
 - e Wait for the solution to clear (about 30 seconds), then carefully remove and discard the supernatant using a pipette set to 120 μL . If necessary, carefully remove any residual liquid with a 20- μL volume pipette.

Step 4. Ligate the captured, circularized fragments

In this step, DNA ligase is added to the capture reaction to close nicks in the circularized HaloPlex probe-target DNA hybrids.

1 Prepare a DNA ligation master mix by combining the reagents in the following table. Calculate the volume needed using the number of reactions in the run plus 1 reaction excess.

Mix the components thoroughly by gentle vortexing then spin the tube briefly.

 Table 6
 Preparation of DNA ligation master mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
Ligation Solution	47.5 μL	807.5 μL
DNA Ligase	2.5 μL	42.5 μL
Total Volume	50 μL	850 µL

- 2 Add 50 μ L of the DNA ligation master mix to the bead-bound DNA in each tube of the Capture Strips.
- 3 Resuspend the beads thoroughly by pipetting up and down 15 times using a 100-μL multichannel pipette set to 40 μL.
- 4 Incubate the tubes in a thermal cycler at 55°C for 10 minutes, using a heated lid.

The thermal cycler may be programmed to include a $4^{\circ}\mathrm{C}$ hold step following the 10-minute incubation.

During the 10-minute incubation, prepare the PCR master mix as specified in the following step.

Step 5. Prepare the PCR Master Mix

Step 5. Prepare the PCR Master Mix

In this step, you prepare a PCR master mix for the captured target DNA amplification step on page 38.

CAUTION

It is critical to include Acetic acid at 2 M concentration in this step to ensure neutralization of the NaOH used for elution on page 37.

See Table 1 on page 9 for 2 M acetic acid supplier information, or prepare 2 M acetic acid from high-quality glacial acetic acid.

1 Prepare the PCR master mix by combining the reagents in the following table.

 Table 7
 Preparation of PCR master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
5X Herculase II Reaction Buffer	20 μL	340 μL
dNTPs (100 mM, 25 mM for each dNTP)	0.8 μL	13.6 μL
Primer 1 (25 μM)	2 μL	34 μL
Primer 2 (25 μM)	2 μL	34 μL
2 M Acetic acid	1 μL	17 μL
Herculase II Fusion DNA Polymerase	2 μL	34 μL
Nuclease-free water	32.2 μL	547.4 μL
Total	60 μL	1020 µL

- **2** Mix the master mix components by gentle vortexing, then distribute 60-µL aliquots to the tubes of fresh 0.2-mL 8-tube strips.
- **3** Store the tubes on ice until they are used in "Step 7. PCR amplify the captured target libraries" on page 38.

Step 6. Elute captured DNA with NaOH

When the 10-minute ligation reaction period is complete, proceed with the following steps to elute the captured DNA libaries.

CAUTION

Using a high-quality NaOH solution for this step is critical for optimal DNA elution and recovery.

Be sure to use freshly-prepared 50 mM NaOH, prepared from 10 M NaOH according to the instructions on page 32.

- 1 Briefly spin the ligation reaction tube strips in a desktop centrifuge and then transfer the tubes to the magnetic plate.
- 2 Wait for the solution to clear (about 30 seconds), then carefully remove and discard the supernatant using a pipette set to 50 μL.
- 3 Remove the tubes from the magnetic plate and add 100 μ L of the SSC Buffer provided with the kit to each tube.
- 4 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-μL multichannel pipette set to 80 μL.
- **5** Briefly spin the tubes and then return the tubes to the magnetic plate.
- **6** Wait for the solution to clear (about 30 seconds), then carefully remove and discard the SSC Buffer using a multichannel pipette set to 120 μ L. If necessary, carefully remove any residual liquid with a 20- μ L volume pipette.
- 7 Add 50 μ L of 50 mM NaOH, which was freshly-prepared on page 32, to each tube.
- 8 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-μL multichannel pipette set to 30 μL.
- **9** Incubate samples for 1 minute at room temperature to allow elution of the captured DNA.
- **10** Briefly spin the tube strips and then transfer to the magnetic plate. Proceed immediately to PCR amplification in the following section.

Step 7. PCR amplify the captured target libraries

Step 7. PCR amplify the captured target libraries

- 1 Prepare amplification reactions by transferring 40 μ L of cleared supernatant from each tube on the magnetic plate to a PCR Master Mix tube held on ice (from page 36).
- 2 Mix by gentle vortexing and then spin briefly to collect the liquid.

 Place the amplification reaction tubes in a thermal cycler and run the program in Table 8, using a heated lid.

Consult the Certificate of Analysis provided with Box 1 of the HaloPlex Exome Target Enrichment System for the PCR cycle number recommendation.

 Table 8
 HaloPlex post-capture DNA amplification PCR program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	Obtain cycle number	98°C	30 seconds
	from Certificate of Analysis	60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	8°C	Hold

Stopping Point

If you do not continue to the next step, PCR products may be stored at -20°C for up to 72 hours or at 8°C overnight. For best results, however, purify PCR products as soon as possible.

Step 8. Purify the amplified target libraries

In this step, the amplified target DNA is purified using AMPure XP beads in two serial rounds of purification.

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes.
- 2 Prepare 800 μL of 70% ethanol per sample, plus excess, for use in step 10.
- 3 Transfer 40 μ L of each PCR reaction sample to the tubes of a fresh 0.2-mL tube strip. Store the remaining volume of each sample at -20°C for troubleshooting.

NOTE

Only 40 μ L of the amplified library DNA sample is purified for sequencing at this time. Do not use a greater volume of the 100- μ L PCR-amplified sample in the purification reaction.

- **4** Mix the AMPure XP bead suspension well, until the suspension appears homogeneous and consistent in color.
- 5 For each sample to be purified, prepare a bead mix by combining 80 μ L of nuclease-free water and 200 μ L of the homogeneous AMPure XP bead suspension. Mix well, until the bead mix suspension appears homogeneous.
- 6 Add 140 μL of the homogeneous bead suspension prepared in step 5 to each 40- μL amplified library sample. Vortex thoroughly.
 - Using this bead-to-sample volume ratio is imperative to ensure optimal purification results.
- 7 Incubate samples for 5 minutes at room temperature with continuous shaking.
 - Make sure the samples are properly mixing in the wells during the 5-minute incubation.
- **8** Spin briefly to collect the liquid, then place the tubes in the magnetic plate. Wait for the solution to clear (approximately 5 minutes).
- **9** Keep the tubes in the magnetic plate. Carefully remove and discard the cleared solution from each tube using a 200- μ L pipette set to 180 μ L. Do not touch the beads while removing the solution.
- 10 Continue to keep the tubes in the magnetic plate while you add 200 μL of 70% ethanol into the tubes.
 - Use fresh 70% ethanol for optimal results.

Step 8. Purify the amplified target libraries

- 11 Wait for 30 seconds to allow any disturbed beads to settle, then remove the ethanol using a 200-µL pipette set to 200 µL.
- **12** Repeat step 10 and step 11 once for a total of two washes.
- 13 Remove any residual ethanol with a 20-µL volume pipette.
- **14** Air-dry the tubes with open lids at room temperature until the residual ethanol completely evaporates.
 - Make sure all ethanol has evaporated before continuing.
- 15 Remove tubes from the magnetic plate and add $50 \mu L$ of 10 mM Tris-acetate or Tris-HCl buffer (pH 8.0) to each sample.

NOTE

Use room-temperature Tris-acetate or Tris-HCl buffer for elution at this step.

- **16** Mix thoroughly by pipetting up and down 15 times using a 100- μ L pipette set to 30 μ L.
- 17 Incubate for 2 minutes at room temperature to allow elution of DNA.
- **18** Put the tube in the magnetic plate and leave for 2 minutes or until the solution is clear.
- 19 Transfer 40 μ L of cleared supernatant to the tubes of a fresh 0.2-mL tube strip. You can discard the beads at this time.
- **20** Repeat step 6 to step 19 once for a total of two rounds of purification of the amplified enriched library samples.

Stopping Point

If you do not continue to the next step, samples may be stored at -20° C for long-term storage (up to one year). Avoid subjecting the stored DNA samples to multiple freeze-thaw cycles.

Step 9. Validate enrichment and quantify enriched target DNA

Prior to sample pooling and sequencing sample preparation, validate enrichment and quantify the enriched target DNA in each library sample by microfluidics analysis using the 2100 Bioanalyzer (see page 42) or the 2200 TapeStation (see page 43).

Enriched library samples may also be qualitatively analyzed using gel electrophoresis. Sample gel electrophoresis results are provided in the Reference section on page 65.

Expected Results

Each amplicon in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform. Amplicons include 50 to 500 bp of target DNA insert and 125 bp of sequencing motifs, as shown in Figure 7.

Figure 7 Content of HaloPlex-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (yellow), the sample index (green) and the library bridge PCR primers (gray).

The amplicons should range from 175 to 625 bp in length and amplicons in the 175 to 625 bp size range should be included for quantitation of the enriched target DNA in each sample. Any spurious DNA products outside of this size range in any sample should be excluded from the target DNA quantitation results.

Option 1: Analysis using the 2100 Bioanalyzer

Use a Bioanalyzer High Sensitivity DNA Assay kit and the 2100 Bioanalyzer with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer instrument and assay setup instructions.

- 1 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μ L of enriched library sample for the analysis.
- **2** Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- **3** Analyze the electropherogram for each sample using the analysis guidelines on page 44.

See Figure 8 for a sample Bioanalyzer system electropherogram.

NOTE

If the concentration determined by Bioanalyzer analysis is > 10 ng/ μ L, repeat the analysis using a 1:10 dilution of the sample. Dilute 1 μ L of the sample in 9 μ L of 10 mM Tris, 1 mM EDTA and then mix well by vortexing at 2000 rpm on the IKA vortex supplied with the Bioanalyzer before analyzing the diluted sample.

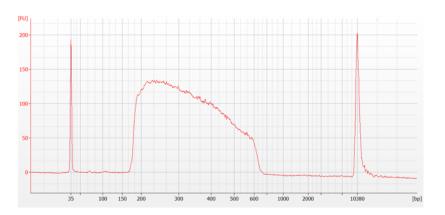


Figure 8 Validation of HaloPlex enrichment by 2100 Bioanalyzer analysis.

Option 2: Analysis using the 2200 TapeStation

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the enriched library samples. For more information to do this step, see the *2200 TapeStation User Manual*.

1 Prepare the TapeStation samples as instructed in the 2200 TapeStation User Manual. Use 2 μ L of each enriched library sample diluted with 2 μ L of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.

CAUTION

Make sure that you thoroughly mix the combined DNA sample and High Sensitivity D1000 sample buffer on a vortex mixer for 5 seconds for accurate results.

- **2** Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the 2200 TapeStation User Manual. Start the run.
- **3** Analyze the electropherogram for each sample using the analysis guidelines on page 44.

See Figure 9 for a sample TapeStation electropherogram.

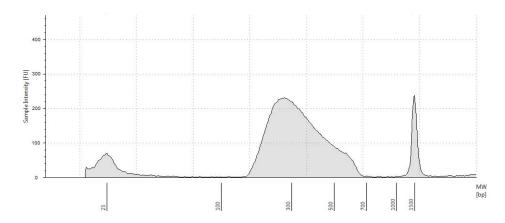


Figure 9 Validation of HaloPlex enrichment by 2200 TapeStation analysis.

Step 9. Validate enrichment and quantify enriched target DNA

Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 175 to 625 bp.
- Determine the concentration of enriched target DNA in the sample by integration under the peak between 175 and 625 bp. Peaks at <150 bp may be observed, but should be excluded from quantitation.

Step 10. Pool samples with different indexes for multiplexed sequencing

Use the following guidelines to design your sample pooling and sequencing strategy:

- Use the Bioanalyzer- or TapeStation-measured concentration of 175-625 bp products in each sample to pool equimolar amounts of differentially indexed samples in order to optimize the use of sequencing capacity.
- The final HaloPlex enrichment pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry on the Illumina HiSeq, MiSeq, or GAIIx platform. See additional guidelines for the MiSeq platform (below) and HiSeq platform (page 49).
- Use 100 + 100 bp read length paired-end sequencing.
- Sequencing runs must be set up to perform an 8-nt index read. For complete index sequence information, see the Reference chapter, starting on page 51.
- Before aligning reads to the reference genome, trim the reads from Illumina adaptor sequences.

MiSeq platform sequencing run setup guidelines

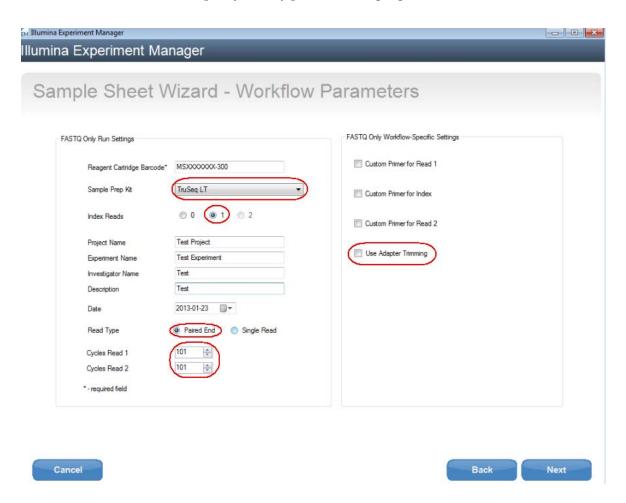
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the HaloPlex indexes used for each sample. For complete index sequence information, see the Reference chapter, starting on page 51.

Setting up a custom Sample Sheet:

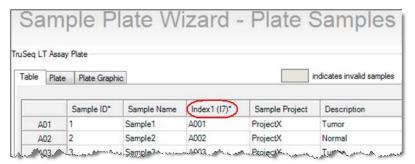
- 1 In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.
 - Under Category, select Other.
 - Under Application, select FASTQ Only.

Step 10. Pool samples with different indexes for multiplexed sequencing

2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below:



3 Using the **Sample Plate Wizard**, set up a New Plate, entering the required information for each sample to be sequenced. In the **Index** 1(17) column of the **TrueSeq LT Assay Plate** table, assign each sample to any of the Illumina 17 indexes. The index will be corrected to a HaloPlex index at a later stage.



- **4** Finish the sample plate setup tasks and save the sample plate file.
- **5** Using the **Sample Sheet Wizard**, select the samples to include in the run and save the Sample Sheet file.

Step 10. Pool samples with different indexes for multiplexed sequencing

Editing the Sample Sheet to include HaloPlex indexes:

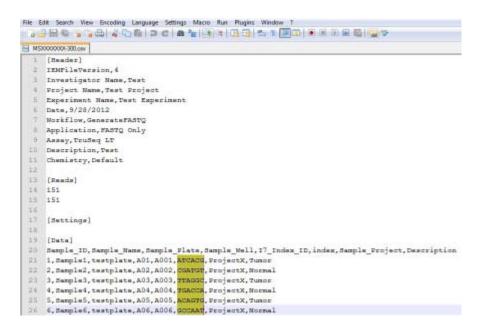
CAUTION

This guide includes information for kits containing two different sets of indexing primers. Verify that you are referencing the information appropriate for your kit version before you proceed.

Kits with indexing primers supplied in a blue plate include indexing primers A01–H12. See page 55 for index nucleotide sequence information.

Kits with indexing primers supplied in a clear plate include indexing primers 1–96. See page 59 through page 64 for index nucleotide sequence information.

1 Open the Sample Sheet file in a text editor. For each sample, select the text for the 6-nucleotide index (highlighted below), and replace with the appropriate 8-nucleotide HaloPlex index sequence.



2 Save the edited Sample Sheet in an appropriate file location for use in the MiSeq platform run.

HiSeq platform sequencing run setup guidelines

Set up sequencing runs to perform an 8-nt index read using the *Cycles* settings shown in Table 9. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

 Table 9
 HiSeq platform Run Configuration screen Cycle Number settings*

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
Index 2 (i5)	0
Read 2	100

^{*} Settings apply to v3.0 SBS chemistry.

Sequence analysis resources

Agilent's SureCall data analysis software is available to simplify the sequencing data analysis workflow after HaloPlex target enrichment. To learn more about this resource and download the SureCall software free of charge, visit www.agilent.com/genomics/surecall.

Step 10. Pool samples with different indexes for multiplexed sequencing



Reference Information for Kits with Revised Index Configuration (indexing primers in blue plate or white-capped tubes) 52

Reference Information for Kits with Original Index Configuration (indexing primers in clear plate or clear-capped tubes) 56

Qualitative analysis of enrichment by gel electrophoresis (Optional) 65

This chapter contains reference information, including component kit contents, index sequences, and optional gel validation instructions.

CAUTION

This chapter contains two sets of index sequence and kit content information. Verify that you are referencing the information appropriate for your kit version before you proceed.

The first section covers kits with reconfigured indexing primers, typically received December, 2014 or later. The reconfigured primers A01—H12 are supplied in a blue plate for 96-reaction kits or in white-capped tubes for 16-reaction kits. See page 52 through page 55 for details.

The second section covers kits with original indexing primer configuration, typically received before December, 2014. The original configuration includes primers 1-96, supplied in a clear plate for 96-reaction kits or in clear-capped tubes for 16-reaction kits. See page 56 through page 64 for details.

Reference Information for Kits with Revised Index Configuration (indexing primers in blue plate or white-capped tubes)

Reference Information for Kits with Revised Index Configuration (indexing primers in blue plate or white-capped tubes)

If your kit includes indexing primers in a clear plate or clear-capped tube format, instead see page 56 for kit content and indexing primer information.

Kit Contents-Revised Configuration

The HaloPlex Exome Target Enrichment System (revised index configuration) includes the component kits listed below:

 Table 10
 HaloPlex Exome Target Enrichment System Kit Contents

Kit Type	HaloPlex Exome, ILM, Box 1*	HaloPlex Magnetic Beads, Box 2		
	Store at –20°C	Store at +4°C		
HaloPlex Exome, ILM, 16 Reactions	5190-8062	5190-5383		
HaloPlex Exome, ILM, 96 Reactions	5190-8063	5190-5386		

^{*} See Table 11 for list of included reagents.

The contents of the HaloPlex Exome Target Enrichment System Box 1 (revised index configuration) included with each kit are detailed in the table below:

 Table 11
 HaloPlex Exome Target Enrichment System Box 1 Contents

Included Reagents	16 Reaction Kit	96 Reaction Kit
Hybridization Solution	tube with clear cap	bottle
Ligation Solution	tube with clear cap	bottle
Wash Solution	tube with clear cap	bottle
Capture Solution	tube with clear cap	bottle
SSC Buffer	tube with clear cap	bottle
RE Buffer	tube with clear cap	bottle
BSA Solution	tube with clear cap	tube with clear cap
DNA Ligase	tube with red cap	tube with red cap
Enrichment Control DNA	tube with orange cap	tube with orange cap
Primer 1	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	tube with blue cap
HaloPlex Indexing Primer Cassettes	16 tubes containing Indexing Primer Cassettes A01 to H02 (white-capped tubes)	96-well plate with Indexing Primer Cassettes A01 to H12 (blue plate)
Enzyme Strip 1	8-well strip tube with green label	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label
HaloPlex Probe 8-well Strip	8-well strip tube with black label	8-well strip tube with black label

^{*} See Table 12 for a plate map.

Kit Contents-Revised Configuration

 Table 12
 Plate map for HaloPlex Indexing Primer Cassettes A01 through H12 provided in blue plate with 96-reaction kits

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
В	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
С	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
Н	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of HaloPlex Indexes (indexing primers in blue plate or white-capped tubes)

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex Indexing Primer Cassette (revised index configuration) is provided in the table below.

Table 13 HaloPlex Indexes, for indexing primers provided in blue 96-well plate or white-capped tubes

Index	Sequence	Index	Sequence	Index	Sequence	Inde	x Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10) AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10) TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10) AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10) AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10) CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10) TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10) CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10) ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A1 ⁻	I CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B1	I TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C1	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D1°	I CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G1°	I AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H1°	I AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A1:	2 CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B1:	2 TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	2 CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D1:	2 AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	2 ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	2 GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G1:	2 AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H1:	2 ACAAGCTA

Reference Information for Kits with Original Index Configuration (indexing primers in clear plate or clear-capped tubes)

Reference Information for Kits with Original Index Configuration (indexing primers in clear plate or clear-capped tubes)

If your kit includes indexing primers in a blue plate or white-capped tube format, instead see page 52 for kit content and indexing primer information.

Kit Contents-Original Configuration

The HaloPlex Exome Target Enrichment System (original index configuration) includes the following component kits:

Table 14 HaloPlex Exome Kit Contents

Kit Type	HaloPlex Exome, ILM, Box 1*	HaloPlex Magnetic Beads, Box 2		
	Store at –20°C	Store at +4°C		
HaloPlex Exome, ILM, 16 Reactions	5190-6289	5190-5383		
HaloPlex Exome, ILM, 96 Reactions	5190-6290	5190-5386		

^{*} See Table 15 for list of included reagents.

The contents of the HaloPlex Exome Target Enrichment System Box 1 (original index configuration) included with each kit are detailed in the table below:

 Table 15
 HaloPlex Exome Target Enrichment System Box 1 Contents

Included Reagents	16 Reaction Kit	96 Reaction Kit
Hybridization Solution	tube with clear cap	bottle
Ligation Solution	tube with clear cap	bottle
Wash Solution	tube with clear cap	bottle
Capture Solution	tube with clear cap	bottle
SSC Buffer	tube with clear cap	bottle
RE Buffer	tube with clear cap	bottle
BSA Solution	tube with clear cap	tube with clear cap
DNA Ligase	tube with red cap	tube with red cap
Enrichment Control DNA	tube with orange cap	tube with orange cap
Primer 1	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	tube with blue cap
HaloPlex Indexing Primer Cassettes	16 tubes containing Indexing Primer Cassettes 1-16 (clear-capped tubes)	96-well plate with Indexing Primer Cassettes 1-96 (clear plate) *
Enzyme Strip 1	8-well strip tube with green label	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label
HaloPlex Probe 8-well Strip	8-well strip tube with black label	8-well strip tube with black label

^{*} See Table 16 for a plate map.

Kit Contents-Original Configuration

 Table 16
 Plate map for HaloPlex Indexing Primer Cassettes 1-96 provided in clear plate with 96-reaction kits

	1	2	3	4	5	6	7	8	9	10	11	12
A	Index											
	1	9	17	25	33	41	49	57	65	73	81	89
В	Index											
	2	10	18	26	34	42	50	58	66	74	82	90
С	Index											
	3	11	19	27	35	43	51	59	67	75	83	91
D	Index											
	4	12	20	28	36	44	52	60	68	76	84	92
E	Index											
	5	13	21	29	37	45	53	61	69	77	85	93
F	Index											
	6	14	22	30	38	46	54	62	70	78	86	94
G	Index											
	7	15	23	31	39	47	55	63	71	79	87	95
Н	Index											
	8	16	24	32	40	48	56	64	72	80	88	96

Nucleotide Sequences of HaloPlex Indexes (indexing primers in clear plate or clear-capped tubes)

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex Indexing Primer Cassette (original index configuration) is provided in the tables below. HaloPlex 16-reaction kits include tubes containing the 16 primers listed in Table 17. The 96-reaction kits include plates containing the 96 indexes listed in Table 17 to Table 22.

Table 17 HaloPlex Indexes 1-16

Index Number	Sequence
1	AACGTGAT
2	AAACATCG
3	ATGCCTAA
4	AGTGGTCA
5	ACCACTGT
6	ACATTGGC
7	CAGATCTG
8	CATCAAGT
9	CGCTGATC
10	ACAAGCTA
11	CTGTAGCC
12	AGTACAAG
13	AACAACCA
14	AACCGAGA
15	AACGCTTA
16	AAGACGGA

Nucleotide Sequences of HaloPlex Indexes (indexing primers in clear plate or clear-capped tubes)

 Table 18
 HaloPlex Indexes 17-32

Index Number	Sequence
17	AAGGTACA
18	ACACAGAA
19	ACAGCAGA
20	ACCTCCAA
21	ACGCTCGA
22	ACGTATCA
23	ACTATGCA
24	AGAGTCAA
25	AGATCGCA
26	AGCAGGAA
27	AGTCACTA
28	ATCCTGTA
29	ATTGAGGA
30	CAACCACA
31	CAAGACTA
32	CAATGGAA

 Table 19
 HaloPlex Indexes 33-48

Index Number	Sequence
33	CACTTCGA
34	CAGCGTTA
35	CATACCAA
36	CCAGTTCA
37	CCGAAGTA
38	CCGTGAGA
39	CCTCCTGA
40	CGAACTTA
41	CGACTGGA
42	CGCATACA
43	CTCAATGA
44	CTGAGCCA
45	CTGGCATA
46	GAATCTGA
47	GACTAGTA
48	GAGCTGAA

Nucleotide Sequences of HaloPlex Indexes (indexing primers in clear plate or clear-capped tubes)

 Table 20
 HaloPlex Indexes 49-64

Index Number	Sequence
49	GATAGACA
50	GCCACATA
51	GCGAGTAA
52	GCTAACGA
53	GCTCGGTA
54	GGAGAACA
55	GGTGCGAA
56	GTACGCAA
57	GTCGTAGA
58	GTCTGTCA
59	GTGTTCTA
60	TAGGATGA
61	TATCAGCA
62	TCCGTCTA
63	TCTTCACA
64	TGAAGAGA

 Table 21
 HaloPlex Indexes 65-80

Index Number	Sequence
65	TGGAACAA
66	TGGCTTCA
67	TGGTGGTA
68	TTCACGCA
69	AACTCACC
70	AAGAGATC
71	AAGGACAC
72	AATCCGTC
73	AATGTTGC
74	ACACGACC
75	ACAGATTC
76	AGATGTAC
77	AGCACCTC
78	AGCCATGC
79	AGGCTAAC
80	ATAGCGAC

Nucleotide Sequences of HaloPlex Indexes (indexing primers in clear plate or clear-capped tubes)

 Table 22
 HaloPlex Indexes 81-96

Index Number	Sequence
81	ATCATTCC
82	ATTGGCTC
83	CAAGGAGC
84	CACCTTAC
85	CCATCCTC
86	CCGACAAC
87	CCTAATCC
88	ССТСТАТС
89	CGACACAC
90	CGGATTGC
91	CTAAGGTC
92	GAACAGGC
93	GACAGTGC
94	GAGTTAGC
95	GATGAATC
96	GCCAAGAC

Qualitative analysis of enrichment by gel electrophoresis (Optional)

Enrichment products may be qualitatively analyzed by gel electrophoresis. Analyze 5 μ L of each enriched library sample (enriched ECD sample or experimental enriched libraries) by electrophoresis on a Novex 6% polyacrylamide TBE pre-cast gel. See page 25 for additional gel analysis protocol recommendations.

Successful enrichment is indicated by the presence of a smear of amplicons from approximately 175 to 625 bp in each enrichment library lane. Low molecular weight (<150 bp) bands may also be visible, but should not be included in enriched sample quantitation. See Figure 10 for a sample gel analysis image.

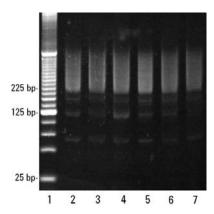


Figure 10 Validation of HaloPlex enrichment process by gel electrophoresis. Lane 1: 25-bp DNA ladder, Lanes 2-7: enriched library samples.

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In This Book

This guide contains information to run the HaloPlex Exome Target Enrichment System protocol for the Illumina sequencing platform.

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