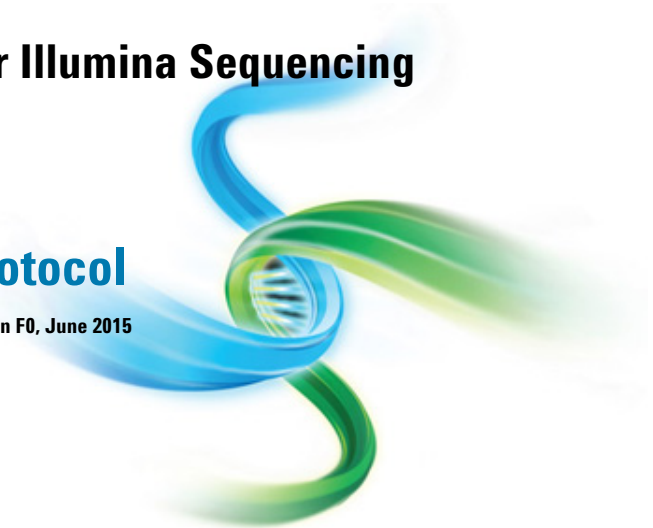
HaloPlex Target Enrichment System

For Illumina Sequencing

Protocol

Version F0, June 2015



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http://www.agilent.com/genomics/protocolvideos.

Notices

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In this Guide...

This guide describes an optimized protocol for using the HaloPlex 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 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 F.0

- Updated product labeling statement

What's New in Version E.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 (48 or 96 Reaction kits). For kit content details see page 50. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 16 on page 53.

  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 (48 or 96 Reaction kits). For kit content details see page 54. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 21 on page 57 through Table 26 on page 62.

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

Content

3 Reference

1 Before You Begin

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 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

CAUTION

- 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 Target Enrichment System Kit	Select the appropriate kit for your probe design from Table 2
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 5 mL 60 mL	Beckman Coulter Genomics p/n A63880 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 500 assays, 2-1000 ng	Life Technologies p/n Q32850 Life Technologies p/n Q32853

* 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).

To select a HaloPlex Target Enrichment System Reagent Kit, use Agilent's SureDesign tool at www.agilent.com/genomics/suredesign to design a custom panel or to select a pre-designed HaloPlex or ClearSeq disease research panel. Reagent kit ordering information is supplied as part of the SureDesign process and is summarized in Table 2 below.

Table 2 HaloPlex Target Enrichment System Kits for Illumina Sequencing

HaloPlex Probe Design	Part Number
Custom Panel Tier 1*, ILM, 96 reactions	G9901B
Custom Panel Tier 1*, ILM, 48 reactions	G9901C
Custom Panel Tier 2†, ILM, 96 reactions	G9911B
Custom Panel Tier 2†, ILM, 48 reactions	G9911C
Custom Panel Tier 3‡, ILM, 96 reactions	G9921B
Custom Panel Tier 3‡, ILM, 48reactions	G9921C
Cancer Research, ILM, 96 reactions	G9903B
Cancer Research, ILM, 16 reactions	G9903A
Cardiomyopathy Research, ILM, 96 reactions	G9908B
Cardiomyopathy Research, ILM, 16 reactions	G9908A
ClearSeq AML, ILM, 96 reactions	G9913B
ClearSeq AML, ILM, 16 reactions	G9913A

* Tier 1 designs are 1-500 kb and up to 20,000 probes.

† Tier 2 designs are 0.5-2.5 Mb OR 1-500 kb with >20,000 probes.

‡ Tier 3 designs are 2.6 Mb-5 Mb.

NOTE Kits contain enough reagents for 96, 48, or 16 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.

Required Equipment

Table 3 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

* 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 4 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 EI0001
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

2 Sample Preparation

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 target region can vary from 1 kb to 5 Mb. Custom HaloPlex probes must be designed before purchasing the kit using Agilent's SureDesign tool at www.agilent.com/genomics/suredesign.

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 up to 96 samples per sequencing lane.

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

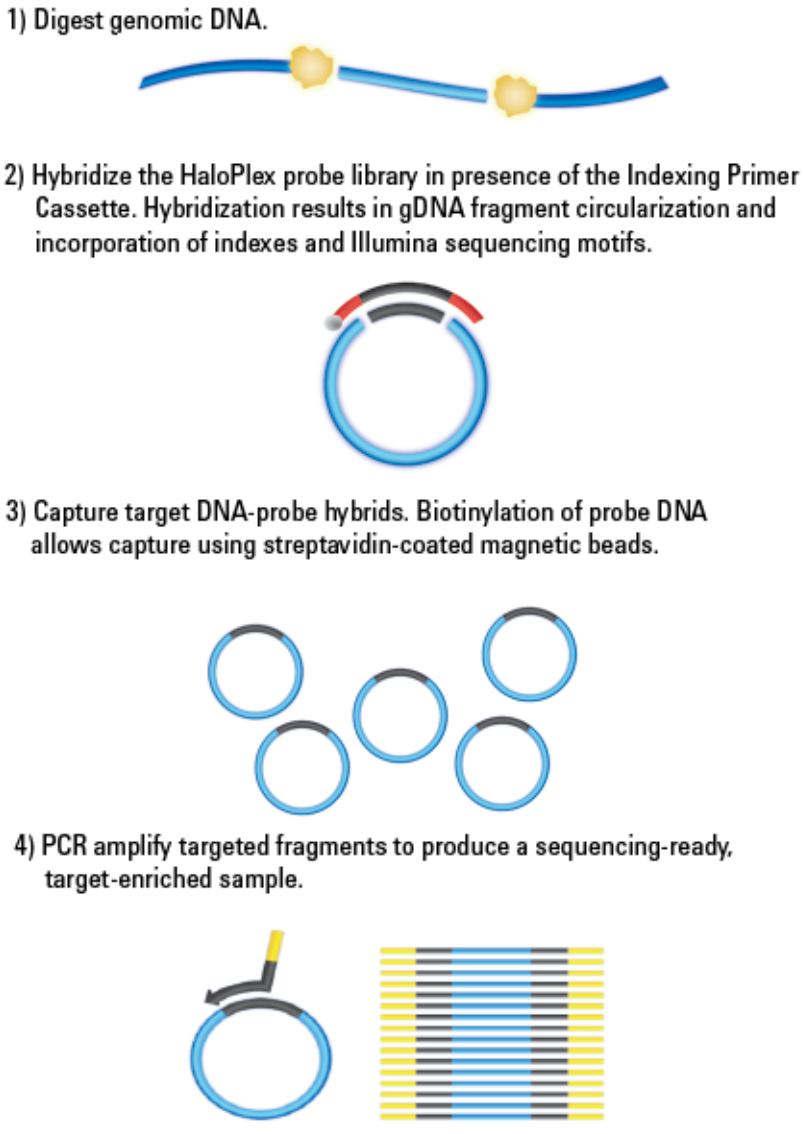


Figure 1 Overall HaloPlex target-enriched sequencing sample preparation workflow.

Run Size Considerations

Kits contain enough reagents for 16, 48 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 12-sample runs. When planning a run size different from 12 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 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 steps and by 25 for hybridization and later steps.

A 96-reaction kit contains enough reagents to prepare master mixes for eight runs of 12 samples each for a total of 96 samples. When processing samples using runs with fewer than 12 samples, some reagents may be depleted before 96 samples are run.

A 48-reaction kit contains enough reagents to prepare master mixes for four runs of 12 samples each, for a total of 48 samples. When processing samples using runs with fewer than 12 samples, some reagents may be depleted before 48 samples are run.

A 16-reaction kit contains enough reagents to prepare master mix 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.

Run Time Considerations

Before you begin, refer to the Certificate of Analysis provided with Box 1 of your kit to determine the hybridization duration appropriate for your design. After reviewing the duration of this and other steps in the protocol, plan the start time for your experiment accordingly.

Designs containing <20,000 probes use a 3-hour hybridization time, and DNA digestion through PCR steps (see Figure 1) are typically run in the same day. Designs containing >20,000 probes use a 16-hour hybridization time, which is typically completed overnight, with the DNA digestion step started in the afternoon.

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.

NOTE

Successful enrichment using the protocol in this guide requires high-quality DNA samples. Before you begin, verify that the genomic DNA samples have 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.

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.

NOTE

Use a fluorometry-based DNA quantitation method, such as Qubit fluorometry or PicoGreen staining, to accurately quantify the DNA starting material.

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.

2 Prepare the DNA samples for the run. For 12-reaction runs, prepare 11 gDNA samples and one Enrichment Control DNA sample.

a In separate 0.2-mL PCR tubes, dilute 225 ng of each gDNA sample in 45 µL nuclease-free water, for a final DNA concentration of 5 ng/µL. Store on ice.

b In a separate 0.2-mL PCR tube, dispense 45 µL of the supplied Enrichment Control DNA (ECD). Store on ice.

NOTE

Although specific instructions are provided for the typical 12-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.

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, along with restriction buffer and BSA to make eight different RE Master Mixes. Figure 2 illustrates how to prepare the 8-well Restriction Enzyme Master Mix strip for a 12-sample run using the steps detailed on page 18.

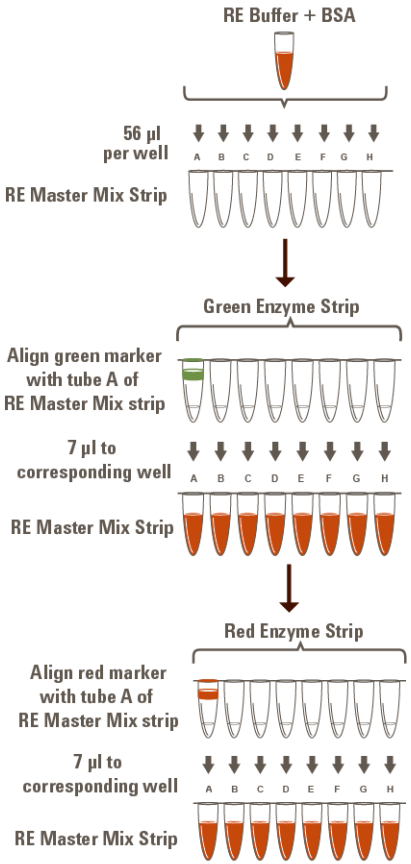


Figure 2 Preparation of the Restriction Enzyme Master Mix Strip for 12-sample run.

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 12 Reactions (includes excess)
RE Buffer	34.0 µL	476 µL
BSA Solution	0.85 µL	11.9 µL
Total Volume	**34.85 µL**	**487.9 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 an 8-well strip tube.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
RE Buffer/BSA mixture	4 µL	56 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 the Green Enzyme Strip, with green marker aligned with tube A, to corresponding tubes A to H of the Restriction Enzyme Master Mix Strip.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
RE Enzymes from Green Enzyme Strip	0.5 µL	7 µL

d Using a multichannel pipette, add the appropriate volume of each enzyme from the Red Enzyme Strip, with red marker aligned with tube A, to each corresponding tube A to H of the same Restriction Enzyme Master Mix Strip.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
RE Enzymes from Red Enzyme Strip	0.5 µL	7 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 of a 96-well plate to be used as the restriction digest reaction plate.

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 µL of each RE master mix row-wise into each well of the plate.

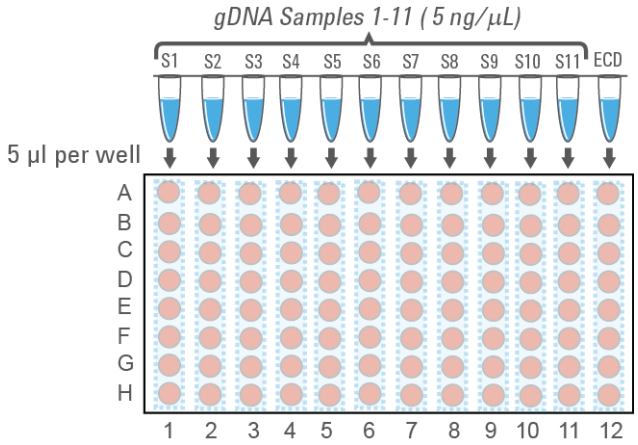
For runs with >12 samples, continue distributing 5 µL from the same RE Master Mix strip row-wise into each well of the additional plates.

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

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

5 Aliquot DNA samples into the 96-well Restriction Digest Reaction Plate(s).

a Align the DNA samples (11 gDNA samples and the ECD sample), prepared in step 2, along the horizontal side of the digestion reaction plate(s) as shown below.



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 plate thoroughly with adhesive plastic film.

6 Carefully vortex the plate to mix the digestion reactions.

7 Briefly spin the plate in a plate centrifuge.

Wells of the prepared 96-well plate 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 the Restriction Digest Reaction Plate in a thermal cycler and run the program in Table 5, using a heated lid.

Table 5 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 at this time. Restriction enzymes are still active and will catalyze inappropriate cleavage events if DNA samples are pooled before enzyme inactivation. DNA samples are pooled during the hybridization step on page 27, upon which restriction enzymes are inactivated by the reaction conditions.

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 Incubate the removed 4-µL samples at 80°C for 5 minutes to inactivate the restriction enzymes.

c 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 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.

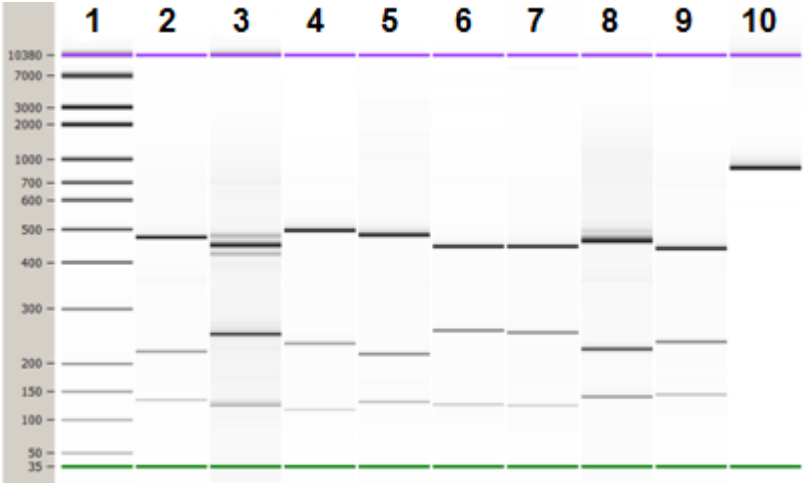


Figure 3 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

Option 2: Validation by 2200 TapeStation analysis

Use a High Sensitivity D1000 ScreenTape and reagent kit. For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

- 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

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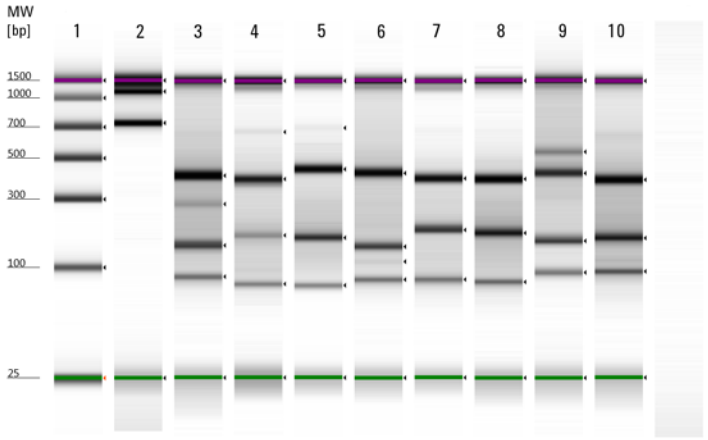


Figure 4 Validation of restriction digestion by 2200 TapeStation analysis. Lane 1: High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

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.

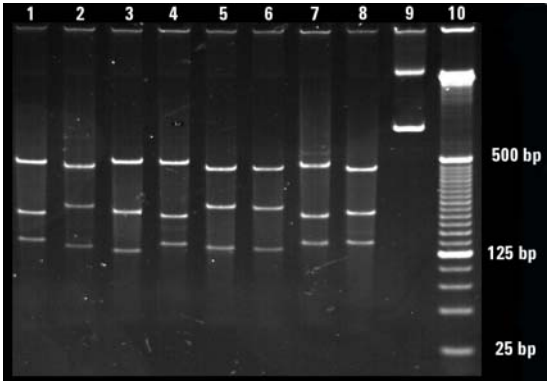


Figure 5 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

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 36.

Step 2. Hybridize digested DNA to HaloPlex probes for target enrichment and sample indexing

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex probe capture library. The duration of the hybridization reaction is determined by the probe density of your design. Refer to the Certificate of Analysis provided with Box 1 of your kit to determine the hybridization conditions appropriate for your design.

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.

1 Prepare a Hybridization Master Mix by combining the reagents in Table 6. Mix well by gentle vortexing, then spin the tube briefly.

Table 6 Hybridization Master Mix

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
Hybridization Solution	50 µL	650 µL
HaloPlex Probe	20 µL	260 µL
Total Volume	**70 µL**	**910 µL**

2 Distribute 70 µL of the Hybridization Master Mix to each of 12 0.2-mL tubes.

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 (typically received December, 2014 or later) include 8-bp indexes A01 through H12. When doing sample indexing primer assignments, see page 50 through page 53 for indexing primer format and nucleotide sequence information.

Kits with indexing primers supplied in clear-capped tubes or clear plate (typically received before December, 2014) include 8-bp indexes 1 through 96. When doing sample indexing primer assignments, see page 54 through page 62 for indexing primer format and nucleotide sequence information.

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

3 Add 10 µL of the appropriate Indexing Primer Cassette to each tube containing Hybridization Master Mix.

Be sure to add only one specific Indexing Primer Cassette to each hybridization tube, using different indexes for each sample to be multiplexed. Record the identity of the Indexing Primer Cassette added to each tube for later sequence analysis.

4 Transfer digested DNA samples from the 96-well Restriction Digest Reaction Plate(s) directly into the hybridization reaction tubes prepared in step 3.

Transfer all eight digestion reactions that correspond to one DNA sample into the appropriate hybridization reaction tube. After addition of each individual digest reaction to the hybridization solution, mix by pipetting before adding the next digest reaction to ensure complete inactivation of the enzymes.

CAUTION

Do **not** pool the digestion samples before adding to the hybridization reaction mixture as restriction enzymes are still active and may catalyze inappropriate cleavage events.

For the ECD sample, add 32 µL of nuclease-free water, in addition to the digested DNA samples, to compensate for the volume removed for digest validation.

After pooling, each hybridization reaction contains the following components:

- 70 µL Hybridization Master Mix
- 10 µL Indexing Primer Cassette
- approximately 80 µL pooled digested DNA samples

NOTE

Due to partial evaporation of samples, you may recover less than 10 µL of each restriction digest. Minor reductions to the digested DNA pool volume will not impact hybridization performance; you do not need to compensate for any sample evaporation volume losses in the final pool.

5 Vortex the mixtures briefly and then spin tubes briefly.

6 Place the hybridization reaction tubes in a thermal cycler. Run the appropriate program in Table 7, using the hybridization duration listed on the Certificate of Analysis.

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 7 Thermal cycler program* for HaloPlex probe hybridization

Step	Temperature	Time (Duration of Step)		
		Disease Research Panels (ClearSeq AML, Cancer Research or Cardiomyopathy Research)	Custom Designs with <20,000 probes (see Certificate of Analysis)†	Custom Designs with >20,000 probes (see Certificate of Analysis)‡
Step 1	95°C	10 minutes	10 minutes	10 minutes
Step 2	54°C	3 hours	3 hours	16 hours

* Thermal cyclers that use calculated temperature methods cannot be set to 160 µL reaction volumes. In that case, enter the maximum possible volume.

† Typical 1-500 kb designs contain <20,000 probes. Please refer to the Certificate of Analysis included with your probe to determine the appropriate hybridization time.

‡ Typical 501 kb-5 Mb designs and some 1-500 kb designs contain >20,000 probes. Please refer to the Certificate of Analysis included with your probe to determine the appropriate hybridization time.

CAUTION

Make sure that the thermal cycler has a maximum reaction volume specification of at least 100 µL.

The 160-µL HaloPlex hybridization reaction conditions have been optimized with the SureCycler thermal cycler (with volume specification of 10-100 µL for PCR reactions). The performance of other thermal cyclers for this application should be verified before use.

Step 3. Capture the target DNA

In this step, the circularized target DNA-HaloPlex probe hybrids, containing biotin, are captured on streptavidin beads.

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 Obtain or prepare 0.5 µL per sample, plus excess, of 2 M acetic acid, for use on page 34.

NOTE

Use a high-quality 2 M acetic acid solution. 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.

3 Prepare 25 µL per sample, plus excess, of fresh 50 mM NaOH for use in the DNA elution step on page 35.

Prepare the 50 mM NaOH solution from a 10M 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 (1 Volume) 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.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
HaloPlex Magnetic Bead suspension	40 µL	520 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 12 Reactions (includes excess)
Capture Solution	40 µL	520 µL

6 Remove the hybridization reactions from the thermal cycler and immediately add 40 µL of the prepared bead suspension to each 160-µL hybridization reaction.

NOTE When adding beads to the hybridization reactions, visually inspect the bead preparation to ensure a homogenous 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 After adding the magnetic beads, mix the capture reactions thoroughly by pipetting up and down 15 times using a 100-µL pipette set to 80 µL.

8 Incubate the capture reactions at room temperature for 15 minutes.

9 Briefly spin the tubes in a desktop centrifuge and then transfer the tubes 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 for samples in PCR tubes or strip tubes.

10 Wait for the solution to clear (about 30 seconds), then remove and discard the supernatant using a pipette set to 200 µL.

11 Wash the bead-bound samples:

a Remove the capture reaction tubes from the magnetic plate and add 100 µL 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 at room temperature 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.

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

Table 8 Preparation of DNA ligation master mix

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
Ligation Solution	47.5 µL	617.5 µL
DNA Ligase	2.5 µL	32.5 µL
Total Volume	**50 µL**	**650 µL**

2 Add 50 µL of the DNA ligation master mix to the beads in each DNA capture reaction tube.

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°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

In this step, you prepare a PCR master mix for the captured target DNA amplification step on page 36.

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 35.

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

Table 9 Preparation of PCR master mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Nuclease-free water	16.1 µL	209.3 µL
5X Herculase II Reaction Buffer	10 µL	130 µL
dNTPs (100 mM, 25 mM for each dNTP)	0.4 µL	5.2 µL
Primer 1 (25 µM)	1 µL	13 µL
Primer 2 (25 µM)	1 µL	13 µL
2 M Acetic acid	0.5 µL	6.5 µL
Herculase II Fusion DNA Polymerase	1 µL	13 µL
Total	**30 µL**	**390 µL**

2 Mix the master mix components by gentle vortexing, then distribute 30-µL aliquots to fresh 0.2-mL reaction tubes.

3 Store the tubes on ice until they are used in "Step 7. PCR amplify the captured target libraries" on page 36.

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 30.

1. Briefly spin the ligation reaction tubes 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 µL 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 µL.

   If necessary, carefully remove any residual liquid with a 20-µL volume pipette.
7. Add 25 µL of 50 mM NaOH, which was freshly-prepared on page 30, to each tube.
8. Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 15 µL.
9. Incubate samples for 1 minute at room temperature to allow elution of the captured DNA.
10. Briefly spin the tubes and then transfer the tubes to the magnetic plate.

    Proceed immediately to PCR amplification in the following section.

Step 7. PCR amplify the captured target libraries

1. Prepare amplification reactions by transferring 20 µL of cleared supernatant from each tube on the magnetic plate to a PCR Master Mix tube held on ice (from page 34).
2. Mix by gentle vortexing and then spin briefly to collect the liquid.
3. Place the amplification reaction tubes in a thermal cycler and run the program in Table 10, using a heated lid.

   The optimal amplification cycle number varies for each HaloPlex Probe design. Consult the Certificate of Analysis (provided with HaloPlex Target Enrichment System Box 1) for the PCR cycling recommendation for your probe.

Table 10 HaloPlex post-capture DNA amplification PCR program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	Obtain cycle number from Certificate of Analysis	98°C	30 seconds
		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.

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 10.
3. Transfer 40 µL of each PCR reaction sample to a fresh 0.2-mL tube. Store the remaining volume of each sample at –20°C for troubleshooting.
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 40 µL of nuclease-free water and 100 µL of the homogenous 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-µL pipette set to 180 µL. 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.
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 40 µL 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-µL pipette set to 30 µL.

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 Remove the cleared supernatant (approximately 40 µL) to a fresh tube. You can discard the beads at this time.

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 microfluidic analysis using the 2100 Bioanalyzer (see page 40) or the 2200 TapeStation (see page 41).

Enriched library samples may also be qualitatively analyzed using gel electrophoresis. Sample gel electrophoresis results are provided in the Reference section on page 63.

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 6.

Figure 6 Content of HaloPlex-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red) and the library bridge PCR primers (yellow).

The amplicons should range from 175 to 625 bp in length, with the majority of products sized 225 to 525 bp. 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 µL 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 42.

See Figure 7 for a sample Bioanalyzer system electropherogram.

NOTE

If the concentration determined by Bioanalyzer analysis is > 10 ng/µL, repeat the analysis using a 1:10 dilution of the sample. Dilute 1 µL of the sample in 9 µL 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.

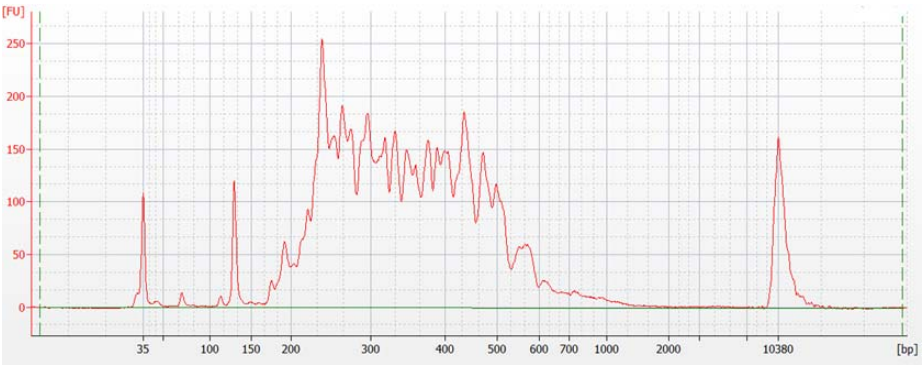


Figure 7 Validation of HaloPlex enrichment by 2100 Bioanalyzer analysis.

Option 2: Analysis using the 2200 TapeStation

Use a High Sensitivity D1000 ScreenTape and reagent kit. For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the *2200 TapeStation User Manual*. Use 2 µL of each enriched library 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 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 42.

See Figure 8 for a sample TapeStation electropherogram.

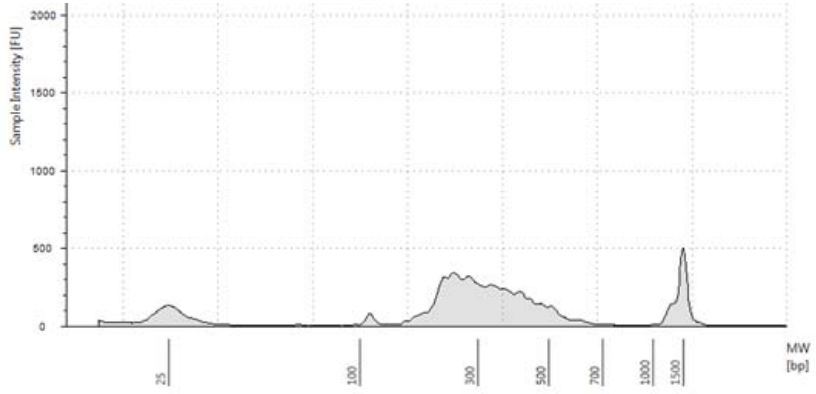


Figure 8 Validation of HaloPlex enrichment by 2200 TapeStation analysis.

Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 225 to 525 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.
- Some designs may generate a peak at about 125 bp. This peak is associated with an adaptor-dimer product which will cluster and generate sequence that does not map to the genome. If the molar fraction of the 125 bp peak is greater than 10%, do another round of AMPure purification after pooling samples. First, pool equimolar amounts of libraries to be multiplexed, using concentrations determined for the 175–625 peak of each sample. Using 40 µL of the pooled libraries, purify the DNA using AMPure XP beads according to the protocol on page 37.

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 47).
- Use 100 + 100 bp or 150 + 150 bp paired-end sequencing, depending on the selection made during probe design. Since the read length affects maximum achievable coverage, check the design report to verify read length selected in probe design.
- 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 49.
- Before aligning reads to the reference genome, trim the reads from Illumina adaptor sequences.

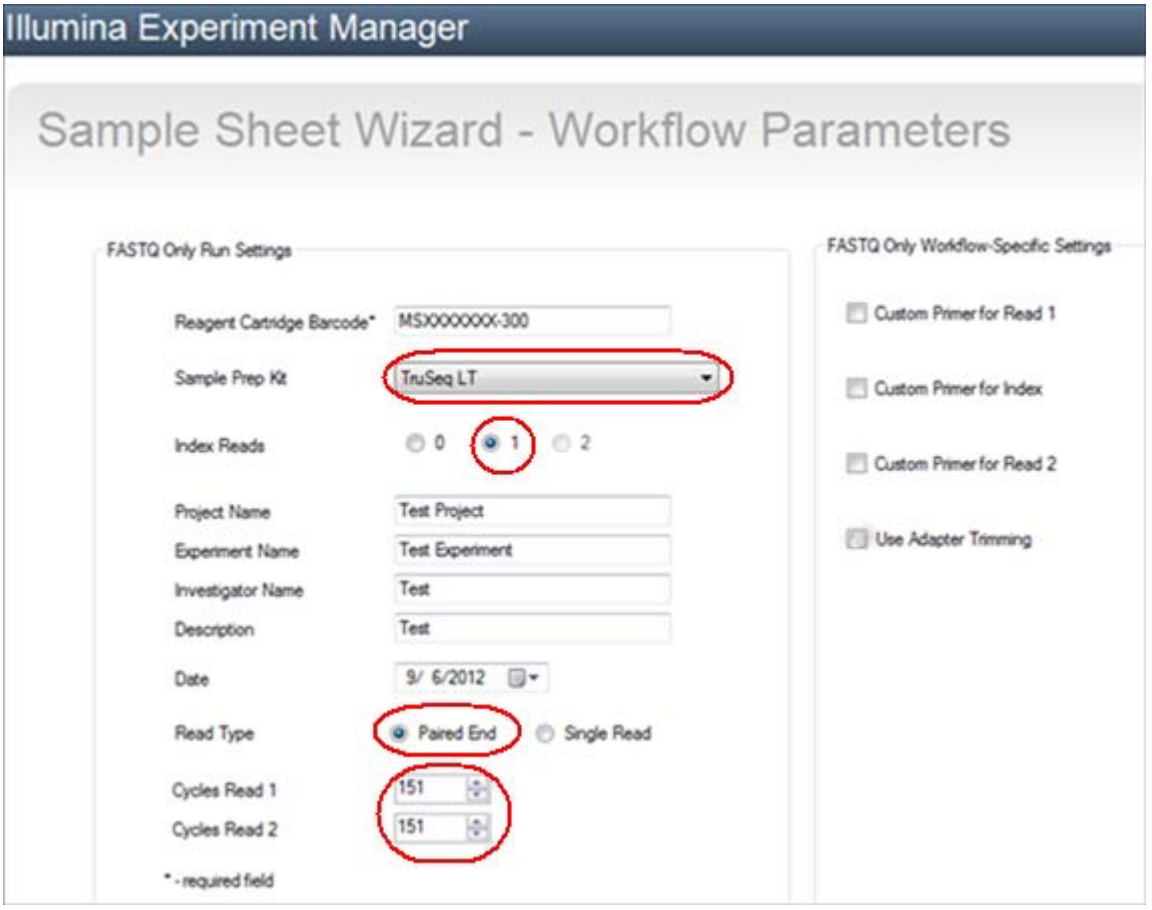
MiSeq platform sequencing run setup guidelines

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. See the Reference chapter, starting on page 49, for nucleotide sequences of the HaloPlex system indexes.

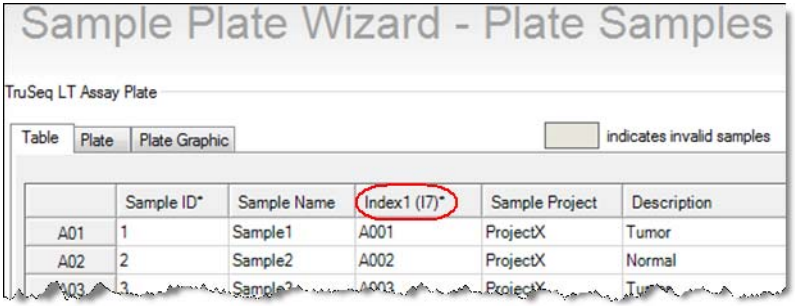
Setting up a custom Sample Sheet:

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*.

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.

Editing the Sample Sheet to include HaloPlex indexes:

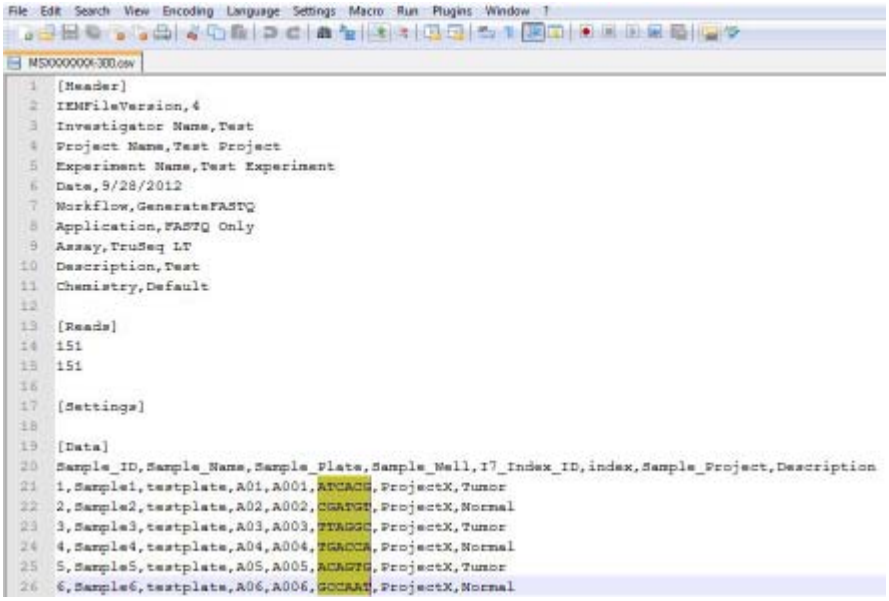
CAUTION

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 53 for index nucleotide sequence information.

Kits with indexing primers supplied in a clear plate include indexing primers 1–96. See page 57 through page 62 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 11. 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 11 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.

3 Reference

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 48- and 96-reaction kits or in white-capped tubes for 16-reaction kits. See page 50 through page 53 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 48- and 96-reaction kits or in clear-capped tubes for 16-reaction kits. See page 54 through page 62 for details.

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 54 for kit content and indexing primer information.

Kit Contents-Revised Configuration

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

Table 12 HaloPlex Target Enrichment System Kit Contents

Design Type	Reaction Number	HaloPlex Target Enrichment System-ILM, Box 1*	HaloPlex Magnetic Beads Box 2
		Store at –20°C	Store at +4°C
Custom 1-500 kb (up to 20,000 probes), ILMFST	**48 Reactions**	5190-8044 **OR** 5190-8045†‡	5190-5976
	96 Reactions	5190-8050 **OR** 5190-8051‡	5190-5386
Custom 0.5-2.5 Mb OR <0.5 Mb with >20,000 probes, ILM	**48 Reactions**	5190-8046 **OR** 5190-8047‡	5190-5976
	96 Reactions	5190-8052 **OR** 5190-8053‡	5190-5386
Custom 2.6 Mb-5 Mb, ILM	**48 Reactions**	5190-8048 **OR** 5190-8049‡	5190-5976
	96 Reactions	5190-8054 **OR** 5190-8055‡	5190-5386
Cancer Research, ILM	**16 Reactions**	5190-8056	5190-5383
	96 Reactions	5190-8057	5190-5386
Cardiomyopathy Research, ILM	**16 Reactions**	5190-8058	5190-5383
	96 Reactions	5190-8059	5190-5386
ClearSeq AML, ILM	**16 Reactions**	5190-8086	5190-5383
	96 Reactions	5190-8087	5190-5386

* See Table 13 for list of included reagents.

† Part number 5190-8044, 5190-8050, 5190-8046, 5190-8052, 5190-8048 or 5190-8054 is provided for the first order of a specific HaloPlex Custom Probe design. Re-order kits, containing previously-purchased Custom Probe designs, include Box 1 part number 5190-8045, 5190-8051, 5190-8047, 5190-8053, 5190-8049 or 5190-8055.

The contents of the HaloPlex Target Enrichment System Box 1 included with each kit are detailed in the table below:

Table 13 HaloPlex Target Enrichment System Box 1 Contents (Revised Index Configuration)

Included Reagents	16 Reaction Kit	48 Reaction Kit	96 Reaction Kit
Hybridization Solution	tube with clear cap	bottle	bottle
Ligation Solution	tube with clear cap	bottle	bottle
Wash Solution	tube with clear cap	bottle	bottle
Capture Solution	tube with clear cap	bottle	bottle
SSC Buffer	tube with clear cap	bottle	bottle
RE Buffer	tube with clear cap	bottle	bottle
BSA Solution	tube with clear cap	tube with clear cap	tube with clear cap
DNA Ligase	tube with red cap	tube with red cap	tube with red cap
Enrichment Control DNA	tube with orange cap	tube with orange cap	tube with orange cap
Primer 1	tube with yellow cap	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	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 H06 (blue plate)*	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	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label	8-well strip tube with red label
HaloPlex or ClearSeq Probe	tube with pink cap	tube with pink cap	tube with pink cap

* See Table 14 for a plate map.

† See Table 15 for a plate map.

Table 14 Plate map for HaloPlex Indexing Primer Cassettes A01 through H06 provided in blue plate with 48-reaction kits; wells in columns 7 through 12 are empty

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	–	–	–	–	–	–
B	B01	B02	B03	B04	B05	B06	–	–	–	–	–	–
C	C01	C02	C03	C04	C05	C06	–	–	–	–	–	–
D	D01	D02	D03	D04	D05	D06	–	–	–	–	–	–
E	E01	E02	E03	E04	E05	E06	–	–	–	–	–	–
F	F01	F02	F03	F04	F05	F06	–	–	–	–	–	–
G	G01	G02	G03	G04	G05	G06	–	–	–	–	–	–
H	H01	H02	H03	H04	H05	H06	–	–	–	–	–	–

Table 15 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
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	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
H	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 16 HaloPlex Indexes, for indexing primers provided in blue 96-well plate or white-capped tubes

Index	Sequence	Index	Sequence	Index	Sequence	Index	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	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

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 50 for kit content and indexing primer information.

Kit Contents-Original Configuration

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

Table 17 HaloPlex Target Enrichment System Kit Contents

Design Type	Reaction Number	HaloPlex Target Enrichment System-ILM, Box 1*	HaloPlex Magnetic Beads Box 2
		Store at –20°C	Store at +4°C
Custom 1-500 kb (up to 20,000 probes), ILMFST	**48 Reactions**	5190-5972 **OR** 5190-5974†‡	5190-5976
	96 Reactions	5190-5385 **OR** 5190-5436‡	5190-5386
Custom 0.5-2.5 Mb OR <0.5 Mb with >20,000 probes, ILM	**48 Reactions**	5190-5977 **OR** 5190-5978‡	5190-5976
	96 Reactions	5190-5534 **OR** 5190-5538‡	5190-5386
Custom 2.6 Mb-5 Mb, ILM	**48 Reactions**	5190-5981 **OR** 5190-5982‡	5190-5976
	96 Reactions	5190-5536 **OR** 5190-5540‡	5190-5386
Cancer Research, ILM	**16 Reactions**	5190-6234	5190-5383
	96 Reactions	5190-6236	5190-5386
Cardiomyopathy Research, ILM	**16 Reactions**	5190-6528	5190-5383
	96 Reactions	5190-6529	5190-5386
ClearSeq AML, ILM	**16 Reactions**	5190-7733	5190-5383
	96 Reactions	5190-7735	5190-5386

* See Table 18 for list of included reagents.

† Part number 5190-5972, 5190-5385, 5190-5977, 5190-5534, 5190-5981 or 5190-5536 is provided for the first order of a specific HaloPlex Custom Probe design. Re-order kits, containing previously-purchased Custom Probe designs, include Box 1 part number 5190-5974, 5190-5436, 5190-5978, 5190-5538, 5190-5982 or 5190-5540.

The contents of the HaloPlex Target Enrichment System Box 1 included with each kit are detailed in the table below:

Table 18 HaloPlex Target Enrichment System Box 1 Contents (Original Index Configuration)

Included Reagents	16 Reaction Kit	48 Reaction Kit	96 Reaction Kit
Hybridization Solution	tube with clear cap	bottle	bottle
Ligation Solution	tube with clear cap	bottle	bottle
Wash Solution	tube with clear cap	bottle	bottle
Capture Solution	tube with clear cap	bottle	bottle
SSC Buffer	tube with clear cap	bottle	bottle
RE Buffer	tube with clear cap	bottle	bottle
BSA Solution	tube with clear cap	tube with clear cap	tube with clear cap
DNA Ligase	tube with red cap	tube with red cap	tube with red cap
Enrichment Control DNA	tube with orange cap	tube with orange cap	tube with orange cap
Primer 1	tube with yellow cap	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	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 1-48 (clear plate)*	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	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label	8-well strip tube with red label
HaloPlex or ClearSeq Probe	tube with pink cap	tube with pink cap	tube with pink cap

* See Table 19 for a plate map.

† See Table 20 for a plate map.

Table 19 Plate map for HaloPlex Indexing Primer Cassettes 1 through 48 provided in clear plate with 48-reaction kits; wells in columns 7 through 12 are empty

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	–	–	–	–	–	–
B	2	10	18	26	34	42	–	–	–	–	–	–
C	3	11	19	27	35	43	–	–	–	–	–	–
D	4	12	20	28	36	44	–	–	–	–	–	–
E	5	13	21	29	37	45	–	–	–	–	–	–
F	6	14	22	30	38	46	–	–	–	–	–	–
G	7	15	23	31	39	47	–	–	–	–	–	–
H	8	16	24	32	40	48	–	–	–	–	–	–

Table 20 Plate map for HaloPlex Indexing Primer Cassettes 1 through 96 provided in clear plate with 96-reaction kits

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	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 clear-capped tubes containing the 16 primers listed in Table 21. The 48- and 96-reaction kits include clear plates containing 48 or 96 indexes listed in Table 21 to Table 26.

Table 21 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

Table 22 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 23 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

Table 24 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 25 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

Table 26 HaloPlex Indexes 81-96

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

Qualitative analysis of enrichment by gel electrophoresis

Enrichment products may be qualitatively analyzed by gel electrophoresis. Analyze 5 µL 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 22 for additional gel analysis protocol recommendations.

Successful enrichment is indicated by the presence of a smear of amplicons from approximately 225 to 525 bp in each enrichment library lane. For some probe designs, low molecular weight (<150 bp) bands may also be visible, but should not be included in enriched sample quantitation. See Figure 9 for a sample gel analysis image.

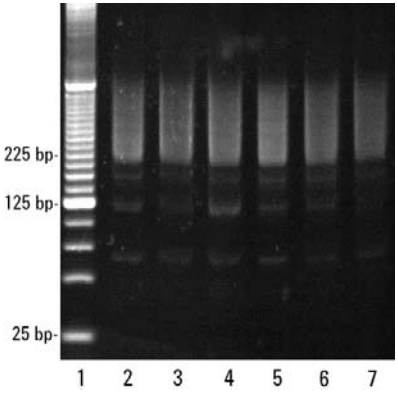


Figure 9 Validation of HaloPlex enrichment process by gel electrophoresis. Lane 1: 25-bp DNA ladder, Lanes 2-7: enriched library samples.

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In This Book

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

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