HaloPlex HS Target Enrichment System

For Illumina Sequencing

Protocol

Version D0, February 2025

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

Manual Part Number

G9931-90000

Edition

Version D0, February 2025

Printed in USA

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In this Guide...

This guide describes an optimized protocol for using the HaloPlex HS 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 HS workflow, to prepare target-enriched sequencing libraries for the Illumina platform.

3 Appendix: Using FFPE-derived DNA Samples

This chapter contains information for optimizing performance when using FFPE-derived DNA samples.

4 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version D0

- Support for kits supplied with re-Albumin Solution, replacing BSA Solution (see page 17, page 18, and page 51). The protocols in this publication are compatible with use of either form of albumin-containing solution.
- Updates to Notices on page 2
- Updates to Technical Support contact information on page 2
- Updates to required materials supplier information in Table 1 on page 8 and Table 3 on page 10
- Updates to lists of available HaloPlex HS kits in Table 2 on page 9 and Table 13 on page 50
- Removal of support information for ClearSeq pre-designed probes throughout this publication
- Updates to purchasing information for the Agilent 2100 Bioanalyzer instrument in Table 4 on page 11, and related updates to validation instructions on page 23 to page 24 and page 41 to page 42
- Updates to information on use of Agilent's SureDesign for access to HaloPlex kit ordering and probe designs information on page 9, page 12 and page 46
- Updates to Agilent.com webpage hyperlinks
- Updates to downstream sequencing support information on page 44 to page 45
- Removal of support information for index plate type no longer provided with this product.

Content

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 HS protocol is optimized for digestion of 50 ng of genomic DNA (split among 8 different restriction digestion reactions) plus excess DNA for pipetting losses. 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 HS Target Enrichment

Description	Vendor and part number
HaloPlex HS Target Enrichment System Kit	Select the appropriate kit for your probe design from Table 2
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
AMPure XP Reagent 5 ml 60 ml	Beckman Coulter Genomics p/n A63880 p/n A63881
Dynabeads MyOne Streptavidin T1 2 mL 10 mL 100 mL	Thermo Fisher Scientific p/n 65601 p/n 65602 p/n 65603
10 M NaOH, molecular biology grade	Sigma, p/n 72068
10 mM Tris-HCl, pH 8.5	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	 Thermo Fisher Scientific p/n Q32850 Thermo Fisher Scientific p/n Q32853
Agilent NGS FFPE QC Kit **(required only for processing FFPE-derived DNA samples)** 16 reactions 96 reactions	Agilent p/n G9700A p/n G9700B

Each HaloPlex HS Target Enrichment System Reagent Kit contains a specific HaloPlex probe. Reagent kit ordering information is provided at Agilent's SureDesign website and is summarized in Table 2 below.

Table 2 HaloPlex HS Target Enrichment System Kits for Illumina Sequencing

	Part Number	
HaloPlex HS Probe Design	**96 Reactions**	**48 Reactions**
Custom Panel Tier 1*, ILMFST	G9931B	G9931C
Custom Panel Tier 2†, ILM	G9941B	G9941C
Custom Panel Tier 3‡, ILM	G9951B	G9951C

* 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, or 48 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 HS Target Enrichment

Description	Vendor and part number
Thermal Cycler	Various suppliers*
Thermal cycler-compatible 96-well plates	Consult the thermal cycler manufacturer's recommendations
8-well strip tubes and caps	Agilent p/n 410092 (strip tubes) and Agilent p/n 410096 (strip tube caps)
12-well strip tubes and caps	Agilent p/n 410082 (strip tubes) and Agilent p/n 410086 (strip tube caps)
96-well plate and strip tube-compatible magnetic separator	DynaMag-96 Side magnet, Thermo Fisher Scientific p/n 12331D, or equivalent
1.5 ml tube-compatible magnetic separator	DynaMag-2 magnet, Thermo Fisher Scientific 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 Fluorometer	Thermo Fisher Scientific p/n Q33226
Qubit assay tubes	Thermo Fisher Scientific 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
Agilent 4200 TapeStation	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA*
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Gel Electrophoresis Platform and Consumables	
XCell SureLock Mini-cell	Thermo Fisher Scientific p/n EI0001
Novex 6% Polyacrylamide, TBE Pre-cast Gels	Thermo Fisher Scientific p/n EC62655BOX
Novex TBE Running Buffer, 5X	Thermo Fisher Scientific p/n LC6675
Novex High-density TBE Sample Buffer, 5X	Thermo Fisher Scientific 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

* The Agilent 2100 Bioanalyzer Instrument is no longer available for sale. Laboratories equipped with this instrument can purchase and use the associated High Sensitivity DNA Kit for the validation steps detailed in this publication.

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. Information on available HaloPlex probe designs can be found at Agilent's SureDesign website using the *Find Designs* tab.

The HaloPlex HS 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. The indexing primers incorporated during hybridization also include degenerate molecular barcode sequences, allowing tracking of individual target amplicons during sequence analysis.

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

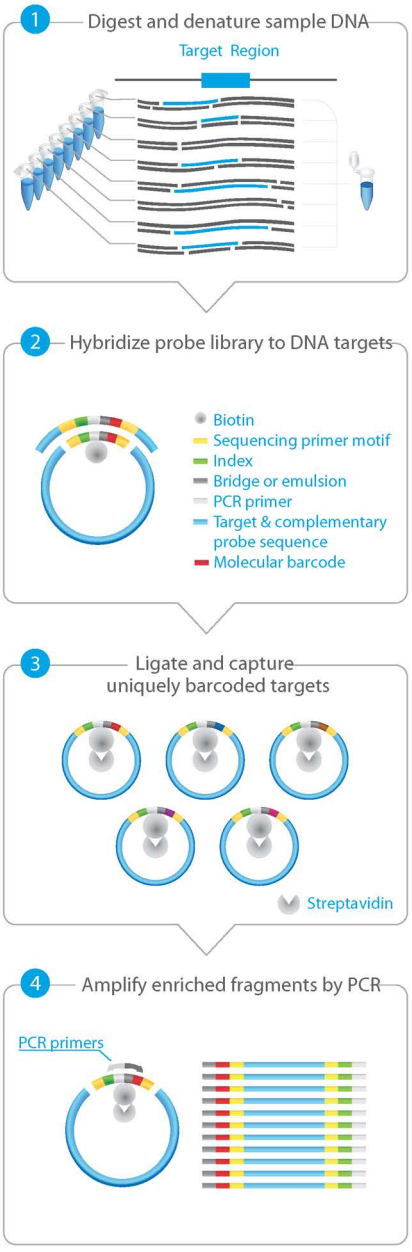


Figure 1 Overall HaloPlex HS target-enriched sequencing sample preparation workflow.

DNA Sample Quality and Quantity Considerations

Target enrichment performance is affected by the quality and precise quantity of the input sample DNA.

Before you begin, verify that 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 standard DNA samples (non-FFPE samples) it is important to use a fluorometry-based DNA quantitation method, such as Qubit fluorometry or PicoGreen staining, as directed in the protocol to accurately quantify the DNA starting material.

In the standard protocol, 50 ng of genomic DNA is split among eight different restriction digests, with additional excess DNA included to allow for pipetting losses (for total DNA input amount of 57.6 ng). Using <50 ng DNA in the enrichment protocol can result in low yield and can potentiate rare allele dropouts.

Target Enrichment from FFPE Samples

This protocol is compatible with FFPE-derived DNA samples after modifying the amount of input DNA according to the DNA quality. Review the Appendix on page 46 before starting the protocol to determine the appropriate amount of input DNA (50 ng or 100 ng) and DNA concentration determination method (fluorometry or qPCR-based).

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 16-sample run, calculate amounts of each solution by multiplying the single reaction value by 18 for restriction digestion steps and by 17 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.

Run Time Considerations

Before you begin, refer to the Certificate of Analysis provided with 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 2-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, in the format of eight double-digests, to create a library of gDNA restriction fragments. Fifty (50) ng of genomic DNA is split among the eight double-digestion reactions, with excess DNA added to allow for pipetting losses (see step 2).

The protocol below presents instructions for a 12-reaction run size, but runs may include up to 96 samples (including one ECD control sample). See page 14 for run size considerations and guidelines for adjusting reagent amounts when using a different run size.

1 Determine the precise DNA concentration for each sample using the fluorometry-based Qubit dsDNA BR Assay or PicoGreen staining kit. Follow the manufacturer's instructions for the kits and instruments.

NOTE For FFPE-derived samples, determine the concentration of amplifiable DNA and assess the DNA quality in each sample using Agilent's qPCR-based NGS FFPE QC Kit.

2 Prepare the DNA samples for the run in a 0.2-ml tube strip. For 12-reaction runs, prepare 11 gDNA samples (see step a and step b below) and one Enrichment Control DNA sample (see step c below).

a Dilute each gDNA sample to concentration of 1.8 ng/µl in 10 mM Tris buffer (pH 8.5).

b Dispense 32 µL of each gDNA sample prepared in step a into the appropriate well of the tube strip. Store the DNA sample strip on ice.

NOTE For FFPE-derived samples, use the guidelines on page 47 to determine the optimal amount of input DNA and optimal method of DNA concentration determination to use at this step.

For FFPE-derived DNA samples that require use of 100 ng DNA in the target enrichment reaction, use 32 µl of a 3.6 ng/µl dilution of the sample DNA for this step.

c Dispense 32 µL of the supplied Enrichment Control DNA (ECD) into a separate well of the tube strip. Store the DNA sample strip on ice until it is used in step 5 on page 20.

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 albumin 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.

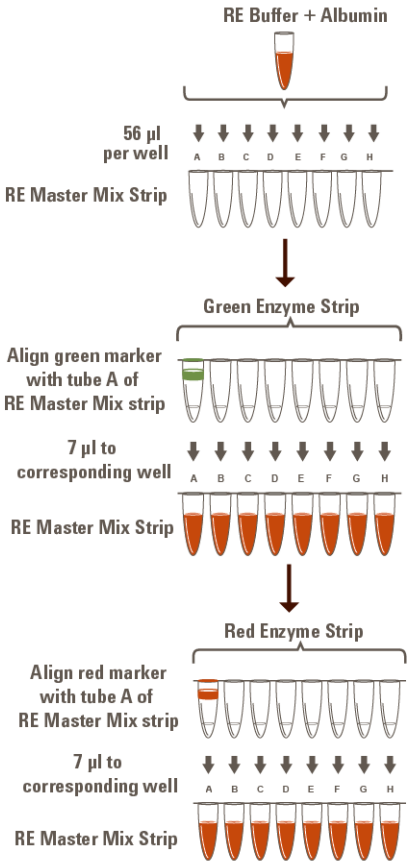


Figure 2 Preparation of the Restriction Enzyme Master Mix Strip for 12-sample run.

a Combine the amounts of RE Buffer and Albumin 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	24.6 µl	344 µl
re-Albumin Solution*	0.64 µl	9 µl
Total Volume	**25.2 µl**	**353 µl**

* re-Albumin Solution replaces the BSA Solution provided with earlier versions of this kit. An equivalent volume of either reagent may be used in this reaction.

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

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
RE Buffer/Albumin mix	2.8 µl	39.2 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	0.35 µl	4.9 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	0.35 µl	4.9 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 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 3.5 µl of each RE master mix row-wise into each well of the plate.

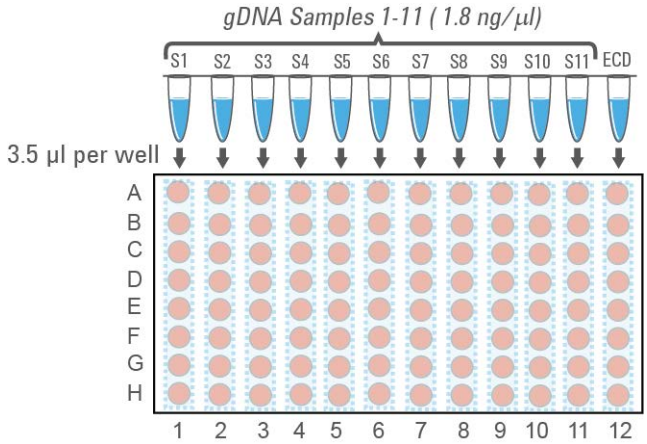
For runs with >12 samples, continue distributing 3.5 µl from the same RE Master Mix strip row-wise into each well of the additional plates.

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

Each row of the 96-well plate now contains 3.5 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 sample strip, prepared in step 2 (containing 11 gDNA samples and the ECD sample), along the horizontal side of the digestion reaction plate as shown below.



b Carefully distribute 3.5 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.

Change tips after pipetting the DNA samples into each digestion reaction mix to prevent cross-contamination of restriction enzymes.

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 7-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 HS restriction digestion

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°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 Briefly spin the digestion reaction plate to collect the digested DNA at the bottom of each well.

b Transfer 4 µl of each ECD digestion reaction from wells of the digestion reaction plate to fresh 0.2-ml PCR tubes.

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

d Analyze the prepared samples by electrophoresis using the Agilent 4200 TapeStation (see page 23), the Agilent 2100 Bioanalyzer (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 4, Figure 3, and Figure 5 does not impact enrichment results.

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

Option 1: Validation by Agilent 4200 TapeStation analysis

Use a High Sensitivity D1000 ScreenTape and reagent kit. See the Agilent High Sensitivity D1000 Assay Quick Guide for assay instructions.

- 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 assay Quick Guide. 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 TapeStation as instructed in the user manual. Start the run.

  See Figure 3 for sample TapeStation electrophoresis results.

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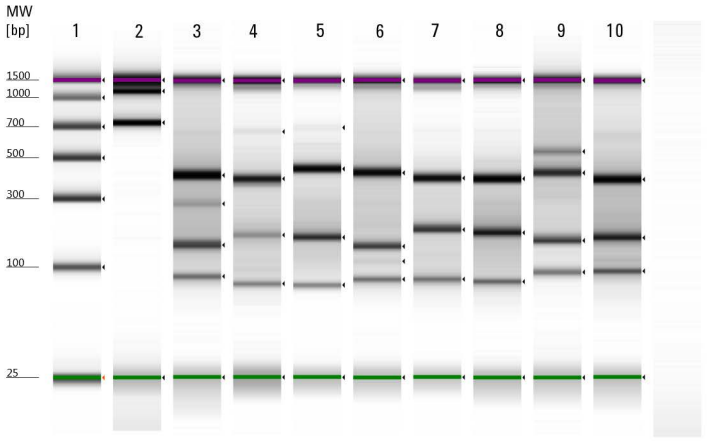


Figure 3 Validation of restriction digestion by TapeStation analysis. Lane 1: High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

Option 2: Validation by 2100 Bioanalyzer analysis

Use a High Sensitivity DNA Kit (p/n 5067-4626) and the 2100 Bioanalyzer system. See the *High Sensitivity DNA Kit Guide* for assay instructions.

- Prepare an undigested DNA gel control by combining 1 µL of the Enrichment Control DNA stock solution and 1 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 dilution 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 4 for sample Bioanalyzer electrophoresis results.

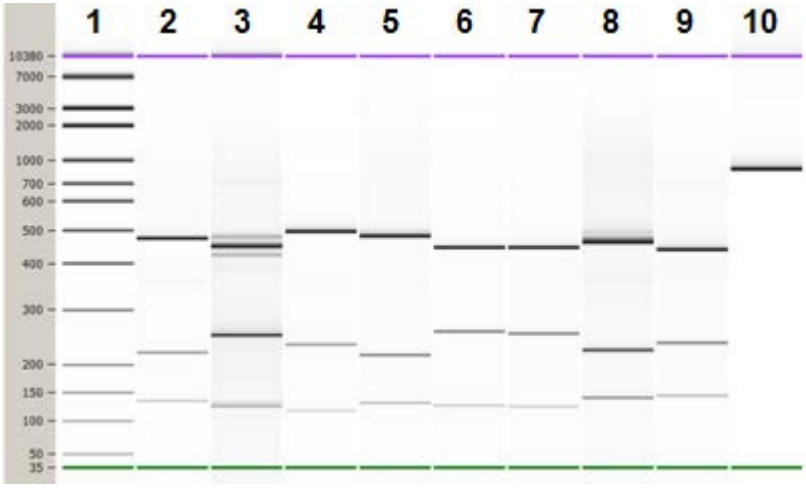


Figure 4 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

Option 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 0.8 µl of the Enrichment Control DNA stock solution and 3.2 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.

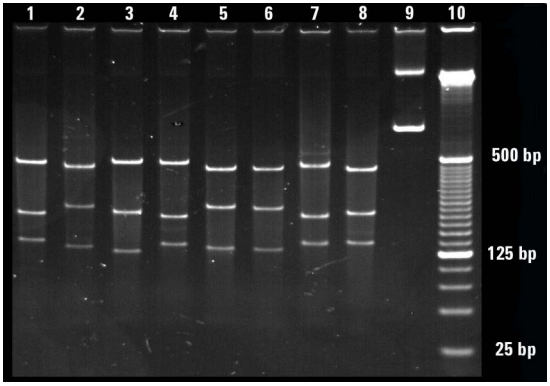


Figure 5 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

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 37.

Step 2. Hybridize digested DNA to HaloPlex HS probes

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex HS probe. During the hybridization process, molecular barcodes and Illumina sequencing motifs, including index sequences, are incorporated into the targeted fragments.

HaloPlex HS probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments. The duration of the hybridization reaction is determined by the probe density of your design. Refer to the Certificate of Analysis provided with your kit to determine the hybridization conditions appropriate for your design.

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	34 µl	442 µl
HaloPlex HS Probe	5 µl	65 µl
Total Volume	**39 µl**	**507 µl**

2 Distribute 39 µl of the Hybridization Master Mix to each of 12 0.2-ml tubes.

3 Add 5 µl of the appropriate HaloPlex HS Indexing Primer to each tube containing Hybridization Master Mix.

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

NOTE

Components needed to incorporate a unique molecular barcode sequence into each target fragment prior to amplification are included in the HaloPlex HS Indexing Primer solutions and do not need to be added separately.

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.

After pooling, each 100-µl hybridization reaction contains the following components:

- 39 µl Hybridization Master Mix
- 5 µl HaloPlex HS Indexing Primer
- approximately 56 µl pooled digested DNA samples

NOTE Due to partial evaporation of samples, you may recover less than 7 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.

For the ECD sample, add 2.5 µL of each digestion reaction and 36 µL of nuclease-free water to the mixture of Hybridization master mix and Indexing Primer from step 3, for a total volume of 100 µL.

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 58°C for more than the indicated time is not recommended.

Table 7 Thermal cycler program for HaloPlex HS probe hybridization

Step	Temperature	Time (Duration of Step)	
		Designs with <20,000 probes (see Certificate of Analysis)	Designs with >20,000 probes (see Certificate of Analysis)
Step 1	95°C	5 minutes	5 minutes
Step 2	58°C	2 hours	16 hours

7 At least 30 minutes before the end of the Hybridization incubation, remove reagents to be used in upcoming protocol steps from cold storage and allow the solutions to reach the appropriate temperature:

- From –20°C storage, remove the HS Hybridization Stop Solution, HS Ligation Solution, HS Capture Solution, HS Wash 1 Solution, HS Wash 2 Solution, and HS Elution Buffer to room temperature.

NOTE

Be sure to bring the HS Hybridization Stop Solution to room temperature before use. The high viscosity of this solution impedes accurate pipetting at lower temperatures.

- From +4°C storage, remove the AMPure XP magnetic beads and the Dynabeads MyOne Streptavidin T1 magnetic beads to room temperature.
- From –20°C storage, remove the 10 mM rATP and HS DNA Ligase to ice.

Step 3. Remove the hybridization buffer

In this step the hybridization buffer is removed, in preparation for the ligation step, using AMPure XP beads.

NOTE The AMPure XP beads and HS Hybridization Stop Solution must be at room temperature for the purification steps. Remove these reagents to room temperature at least 30 minutes before each use.

1. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
2. Mix the AMPure XP bead suspension well, until the suspension appears homogeneous and consistent in color.
3. For each sample to be purified, prepare a bead mix by combining 20 µl of HS Hybridization Stop Solution and 80 µl of the homogeneous AMPure XP bead suspension. Mix well, until the bead suspension mixture appears homogeneous.

NOTE Pipette the viscous HS Hybridization Stop Solution slowly to ensure that the full volume is aspirated and dispensed. Verify that any residual volume of this solution or mixture containing the solution has been dispensed from the pipette tip.

4. Add 100 µL of the homogeneous stop solution bead suspension prepared in step 3 to each 100-µL hybridized library sample. Mix by pipetting up and down 10 times using a 200-µL pipette set to 150 µL.
5. Incubate samples for 5 minutes at room temperature with continuous shaking at 1300 rpm.

   Make sure the samples are properly mixing in the wells during the 5-minute incubation.

NOTE During the 5-minute incubation period, prepare ligation reagents as instructed in step 1 and step 2 on page 31.

6. Spin briefly to collect the liquid, then place the tubes in the magnetic plate. Wait for the solution to clear (approximately 5 minutes).
7. Keep the tubes in the magnetic plate. Carefully remove and discard the cleared solution from each tube. Do not touch the beads while removing the solution.

8 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.

9 Wait for 1 minute to allow any disturbed beads to settle, then remove and discard the ethanol.

10 Repeat step 8 and step 9 once for a total of two washes.

11 Spin the samples briefly, then place the tubes in the magnetic plate and remove any residual ethanol with a 10-µl volume pipette.

12 Air-dry the samples by keeping the tubes with open lids at room temperature until any residual ethanol evaporates (approximately 1–5 minutes).

CAUTION Take care not to over dry the beads. Overdrying makes the beads difficult to resuspend in step 4 on page 31 and decreases elution efficiency.

Step 4. Ligate the circularized fragments

In this step, DNA ligation reagents are added to the circularized hybridization products to close nicks in the probe-target DNA hybrids.

1 Prepare a 1 mM rATP solution by diluting the provided 10 mM rATP 1:10 with nuclease-free water.

For 12-sample runs, combine 1 µl of the provided 10 mM rATP and 9 µl of nuclease-free water.

2 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)
HS Ligation Solution	10 µl	130 µl
1 mM rATP (prepared in step 1)	0.6 µl	7.8 µl
Nuclease-free water	39.4 µl	512.2 µl
Total Volume	**50 µl**	**650 µl**

3 Remove the DNA sample tubes from the magnetic plate (see step 12 on page 30), then add 50 µL of the DNA ligation master mix to each sample.

4 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 40 µL.

Visually inspect the beads to verify complete resuspension; if needed, do additional rounds of pipetting up and down until the beads are in a homogeneous suspension.

5 Incubate the samples for 2 minutes at room temperature to elute the DNA samples from the beads.

6 Spin briefly to collect the liquid, then place the tubes in the magnetic plate. Wait for the solution to clear (approximately 5 minutes).

7 For each sample, transfer 47.5 µl of the cleared supernatant to a fresh tube.

Pipette slowly to minimize transfer of beads with the supernatant.

8 Add 2.5 µl of HS DNA Ligase to each sample tube. Mix by gentle vortexing and then spin briefly to collect the liquid.

9 Incubate the tubes in a thermal cycler at 55°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.

During the 10-minute incubation, prepare the following components for later protocol steps:

- Dynabeads MyOne Streptavidin T1 magnetic beads (prepare as described in step 1 and step 2 on page 33.)
- Wash 1 Mix (prepare as described in step 3 on page 33.)

Step 5. Capture the target DNA

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

1 Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.

2 For each sample in the run prepare 40 µl (1 Volume) of Dynabeads MyOne Streptavidin T1 magnetic beads in HS Capture Solution, using the steps below:

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)
Dynabeads MyOne Streptavidin T1 bead suspension	40 µl	520 µl

b Put the tube into a 1.5 ml tube-compatible magnetic rack until the solution has cleared (approximately 5 minutes).

c Carefully remove and discard the cleared supernatant using a pipette.

d Add an equivalent volume of HS Capture Solution to the beads and resuspend by pipetting up and down.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
HS Capture Solution	40 µl	520 µl

3 Prepare Wash 1 Mix for use on page 35 using the following steps:

a Prepare 10 µL per sample, plus excess, of fresh 1 M NaOH for use in step b.

Prepare the 1 M NaOH solution from a 10 M NaOH stock solution.

CAUTION

Using high-quality NaOH is critical for optimal DNA sample quality.

- Do not use stock NaOH solutions that were stored at concentrations below 10 M to prepare the 1 M NaOH solution.
- Keep the 1 M NaOH solution container sealed when not in use, especially when processing large numbers of samples per run.

b Prepare Wash 1 Mix by combining the reagents in the following table.

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
HS Wash 1 Solution	90 µl	1170 µl
1 M NaOH (prepared in step a)	10 µl	130 µl
Total Volume	**100 µl**	**1300 µl**

4 Remove the ligation reactions from the thermal cycler and immediately add 40 µL of the HS Capture Solution + streptavidin bead mixture (prepared in step 2) to each 50-µL ligation reaction.

NOTE

When adding beads to the ligation reactions, 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.

5 Incubate samples for 15 minutes at room temperature with continuous shaking.

Make sure the samples are properly mixing in the wells during the 15-minute incubation.

During the 15-minute incubation, prepare the PCR Master Mix as described on page 36.

6 Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic plate.

7 Wait for the solution to clear, then remove and discard the supernatant using a pipette.

8 Wash the bead-bound samples:

a Remove the capture reaction tubes from the magnetic plate and add 100 µL of the Wash 1 Mix prepared in step 3 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 then briefly spin the tubes in a desktop centrifuge.

c Incubate the samples at room temperature for 1 minute.

d Transfer the tubes to the magnetic plate. Wait for the solution to clear, then remove and discard the supernatant using a pipette.

e Add 150 µl of the provided HS Wash Solution 2 to each tube.

f Resuspend the beads thoroughly by pipetting up and down 10 times, then briefly spin the tubes in a desktop centrifuge.

g Transfer the tubes to the magnetic plate. Wait for the solution to clear, then remove and discard the supernatant using a pipette.

Step 6. PCR amplify the captured target library

In this step, the captured target libraries are amplified by PCR.

1 Prepare the PCR master mix, on ice, 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	53.2 µl	691.6 µl
Herculase II Reaction Buffer	30 µl	390 µl
dNTPs (100 mM, 25 mM for each dNTP)	0.8 µl	10.4 µl
Primer 1	4 µl	52 µl
Primer 2	8 µl	104 µl
Herculase II Fusion DNA Polymerase	4 µl	52 µl
Total	**100 µl**	**1300 µl**

2 Mix the master mix components by gentle vortexing. Store the mixture on ice until it is used in step 3.

NOTE

The PCR Master Mix is typically prepared during the 15-minute capture step on page 34.

3 Remove the captured and washed DNA sample tubes from the magnetic plate (see step g on page 35), then transfer 100 µL of PCR Master Mix to each sample tube. Mix by pipetting up and down until the beads are in a homogeneous suspension.

4 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 HS probe design. Consult the Certificate of Analysis provided with your kit for the PCR cycling recommendation for your probe.

.

Table 10 HaloPlex HS 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

During amplification, remove the AMPure XP magnetic beads from +4°C storage, and allow the beads to come to room temperature for use on page 38.

5 When the PCR program is complete, briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic plate.

6 Wait for the solution to clear, then remove 40 µl of each PCR reaction sample to a fresh 0.2-ml tube for purification.

Store the remaining volume of each sample at –20°C for troubleshooting.

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 7. Purify the amplified target library

In this step, the amplified target DNA is purified using AMPure XP beads.

1. Verify that the AMPure XP beads have been kept at room temperature for at least 30 minutes.
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 9.
3. Mix the AMPure XP bead suspension well, until the suspension appears homogeneous and consistent in color.
4. For each sample to be purified, prepare a bead mix by combining 40 µl of nuclease-free water and 100 µl of the homogeneous AMPure XP bead suspension. Mix well, until the bead mix suspension appears homogeneous.
5. Add 140 µL of the homogeneous bead suspension prepared in step 4 to each 40-µL amplified library sample. Vortex thoroughly.

   Using this bead-to-sample volume ratio is imperative to ensure optimal purification results.
6. 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.
7. Spin briefly to collect the liquid, then place the tubes in the magnetic plate. Wait for the solution to clear (approximately 5 minutes).
8. 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.
9. 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.
10. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol using a 200-µl pipette set to 200 µl.
11. Repeat step 9 and step 10 once for a total of two washes.
12. Spin the samples briefly, then place the tubes in the magnetic plate and remove any residual ethanol with a 10-µl volume pipette.

13 Air-dry the samples by keeping the tubes with open lids at room temperature until any residual ethanol evaporates (approximately 1–5 minutes).

Make sure all ethanol has evaporated before continuing.

14 Remove tubes from the magnetic plate and add 45 µl of HS Elution Buffer to each sample.

15 Mix thoroughly by pipetting up and down 10 times using a 100-µl pipette set to 30 µl.

16 Incubate the tubes at room temperature for 2 minutes to allow DNA elution.

17 Put the tubes in the magnetic plate and leave for 2 minutes or until the solution is clear.

18 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 8. 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 using the Agilent 4200 TapeStation (see page 41) or 2100 Bioanalyzer (see page 42).

Expected Results

Each amplicon in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 6.

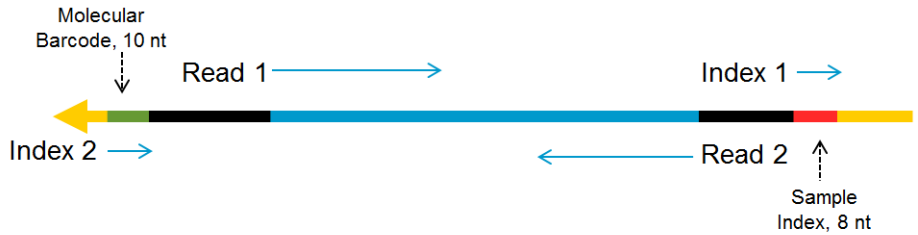


Figure 6 Content of HaloPlex HS-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red), the molecular barcode (green) and the library bridge PCR primers (yellow).

The amplicons should be approximately 190 to 640 bp in length, with the majority of products sized approximately 225 to 540 bp. Although the DNA fragment size distribution may vary for different DNA samples and different probe designs, use the constant size range of 175 to 625 bp for quantitation of the enriched target DNA in each sample. Any spurious DNA products outside of the 175 to 625 bp size range should be excluded from the target DNA quantitation results.

Option 1: Analysis using the Agilent 4200 TapeStation

Use a High Sensitivity D1000 ScreenTape and reagent kit. See the Agilent High Sensitivity D1000 Assay Quick Guide for assay instructions.

1 Prepare the TapeStation samples as instructed in the reagent Quick Guide. 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 4200 TapeStation and start the run.

3 Analyze the electropherogram for each sample using the analysis guidelines on page 42.

See Figure 7 for a sample TapeStation electropherogram.

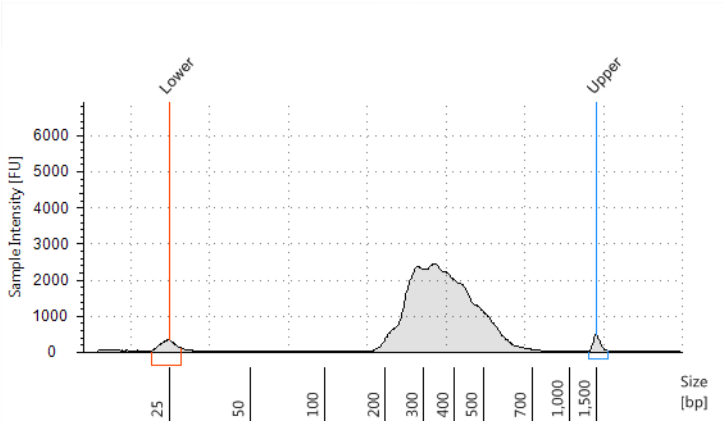


Figure 7 Validation of HaloPlex HS enrichment by TapeStation analysis.

Option 2: Analysis using the 2100 Bioanalyzer

Use a Bioanalyzer High Sensitivity DNA Assay kit and the Agilent 2100 Bioanalyzer. See the *High Sensitivity DNA Kit Guide* for assay 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 8 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.

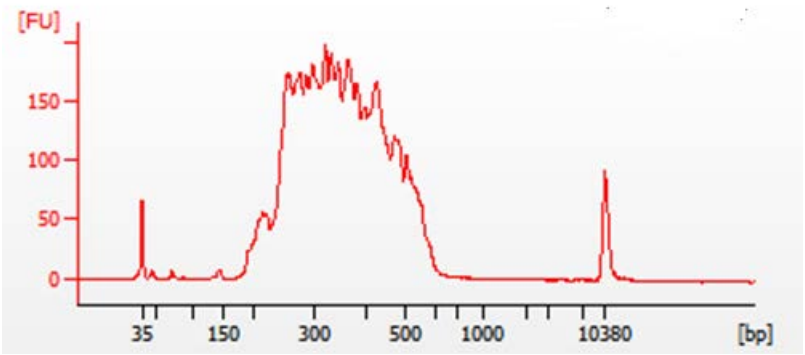


Figure 8 Validation of HaloPlex HS enrichment by 2100 Bioanalyzer analysis.

Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 225 and 540 bp.
- Determine the concentration of enriched target DNA in the sample by integration under the peak between 175 and 625 bp. Peaks at <175 bp may be observed, but should be excluded from quantitation.
- Some designs may generate a peak at about 140 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 140 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 bp peak of each sample. Using 40 µL of the pooled libraries, purify the DNA using AMPure XP beads according to the protocol on page 38.

Step 9. Pool indexed samples and perform multiplexed sequencing

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

- Use the TapeStation- or Bioanalyzer-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 an Illumina platform. When using the MiSeq platform, see page 45 for special instrument setup instructions before your first run. If you need assistance with the NGS segment of the workflow, please contact Agilent's NGS Support team or your local representative.
- 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 sample-level Index 1 (i7) read. For complete index sequence information, see Table 17 on page 53.
- The degenerate molecular barcode requires a 10-nt index (i5) read. Enter NNNNNNNNNN for the i5 degenerate molecular barcode for all samples.

  Some Illumina FASTQ file generation software applications, including MiSeq Reporter and Local Run Manager (v2 and v3), support the use of 'N' wildcard characters in index reads and use of these applications is recommended for run setup and demultiplexing. If your NGS workflow requires a different FASTQ file generation or demultiplexing software application which does not support use of 'N' wildcards in index reads, consult Illumina's resources for application-specific instructions for base masking or cycle override settings to allow use of the N_{10} molecular barcode sequences.
- Before aligning reads to the reference genome, trim the Illumina adaptor sequences from the reads. Note that the first base of Read 2 needs to be manually trimmed, since this base originates from non-target DNA.

MiSeq platform sequencer setup requirements

Before the first use of the MiSeq instrument for HaloPlex HS library sequencing, you must adjust the MiSeq Reporter settings to generate FASTQ files for index reads. Once changed, this setting is retained for future runs.

To change this setting, open the file **MiSeq Reporter.exe.config**. Under the **<appSettings>** tag, add **<add key="CreateFastqForIndexReads" value="1"/>**. You must restart the instrument for this setting change to take effect.

NOTE

If you are using the MiSeqDx platform, run the instrument in research mode to make changes to MiSeq Reporter settings. If research mode is not available on your instrument, you may need to upgrade the system to include the dual boot configuration to allow settings changes in research mode.

3 Appendix: Using FFPE-derived DNA Samples

FFPE-derived DNA samples may be used in HaloPlex HS Target Enrichment protocol after making the minor protocol modifications detailed in this chapter, based on the measured integrity of the FFPE sample DNA.

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample and a ΔΔCq DNA integrity score. HaloPlex HS protocol modifications based on ΔΔCq scores for individual samples are detailed in this chapter.

NOTE

Some HaloPlex HS probes designed in Agilent's SureDesign for use with FFPE-derived samples include the **Optimize for FFPE Samples** design enhancement produced using the *Tile Genes or Regions* option.

The HaloPlex HS protocol modifications and downstream sequencing modifications in this chapter are applicable to all probes designed either with or without using this design enhancement.

HaloPlex HS Protocol Modifications

Before applying protocol modifications in this section, use the Agilent NGS FFPE QC Kit to determine the ΔΔCq normalized DNA integrity score and the quantity of amplifiable DNA for each FFPE DNA sample. The complete Agilent NGS FFPE QC Kit protocol (G9700-90000) is available at agilent.com.

Once the ΔΔCq DNA integrity scores have been determined for each sample, modify the DNA input amount, if needed, as summarized in Table 11.

Table 11 HaloPlex HS protocol modifications based on DNA integrity score

Protocol Step and Parameter	non-FFPE Samples	FFPE Samples		
		ΔΔCq≤1.3	ΔΔCq between 1.3 and 1.5	ΔΔCq between 1.5 and 2.5*
DNA input for Library Preparation	50 ng, based on Qubit Assay	50 ng, based on Qubit Assay	50 ng of amplifiable DNA, based on qPCR quantification	100 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with ΔΔCq values >2.5 may be used for HaloPlex HS Target Enrichment, but enrichment results and sequencing performance may be impacted. For best results, use the same protocol modifications provided for samples with ΔΔCq values between 1.5 and 2.5.

Downstream Sequencing Modifications

Determine the amount of extra sequencing output required for FFPE DNA samples.

1 Find the Total Sequenceable Design Size for your probe in the HaloPlex Design Report.

2 Use the guidelines in Table 12 to determine the recommended amount of sequencing output for each HaloPlex HS-enriched FFPE sample based on the ΔΔCq DNA integrity score.

Table 12 Recommended sequencing augmentation for FFPE-derived DNA samples

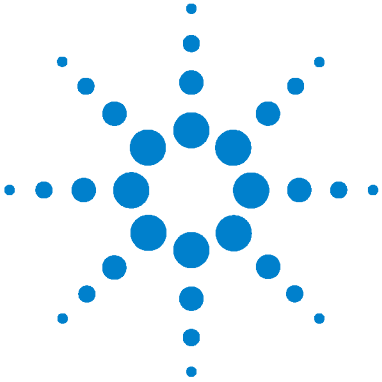
ΔΔCq value	Recommended amount sequencing output for FFPE-derived sample
≤1.3	300–600× Total Sequenceable Design Size
between 1.3 and 1.5	600–1200× Total Sequenceable Design Size
between 1.5 and 2.5*	1200× Total Sequenceable Design Size

* FFPE samples with ΔΔCq values >2.5 may be used for HaloPlex HS Target Enrichment, but enrichment results and sequencing performance may be impacted. For best results, use the same protocol modifications provided for samples with ΔΔCq values between 1.5 and 2.5.

NOTE

A general guideline for non-FFPE samples is to collect sequencing output equivalent to approximately 200× the Total Sequenceable Design Size.

For example, if the Total Sequenceable Design Size is 1 Mb, an FFPE sample with ΔΔCq score of 1.4 requires 600–1200 Mb of sequencing output to achieve coverage similar to that expected for an intact DNA sample with 200 Mb of sequencing output.



4 Reference

Kit Contents

The HaloPlex HS Target Enrichment System is supplied using the part numbers listed below:

Table 13 HaloPlex HS Target Enrichment System Kit Part Numbers

Design Type	Kit Part Number	Component Box Part Number* (Store at –20°C)
Custom 1-500 kb (up to 20,000 probes), ILMFST	**G9931B (96 Reactions)**	5190-7847 **OR** 5190-7849
	G9931C (48 Reactions)	5190-7835 **OR** 5190-7837
Custom 0.5-2.5 Mb OR <0.5 Mb with >20,000 probes, ILM	**G9941B (96 Reactions)**	5190-7851 **OR** 5190-7853
	G9941C (48 Reactions)	5190-7839 **OR** 5190-7841
Custom 2.6 Mb-5 Mb, ILM	**G9951B (96 Reactions)**	5190-7855 **OR** 5190-7857
	G9951C (48 Reactions)	5190-7843 **OR** 5190-7845

* See Table 14 for list of included reagents. Part number 5190-7835, 5190-7847, 5190-7839, 5190-7851, 5190-7843 or 5190-7855 is provided for the first order of a specific HaloPlex HS Custom Probe design. Re-order kits, containing previously-purchased Custom Probe designs, include part number 5190-7837, 5190-7849, 5190-7841, 5190-7853, 5190-7845 or 5190-7857.

The contents of the HaloPlex HS Target Enrichment Kits are detailed in Table 14.

Table 14 HaloPlex HS Target Enrichment System Kit Contents

Included Reagents	48 Reaction Kit	96 Reaction Kit
RE Buffer	tube with clear cap	bottle
re-Albumin Solution*	tube with clear cap	tube with clear cap
Enzyme Strip 1	8-well strip with green label	8-well strip with green label
Enzyme Strip 2	8-well strip with red label	8-well strip with red label
Enrichment Control DNA	tube with orange cap	tube with orange cap
Hybridization Solution	bottle	bottle
HS Hybridization Stop Solution	tube with clear cap	bottle
HS Ligation Solution	tube with black cap	tube with black cap
HS DNA Ligase	tube with green cap	tube with green cap
10 mM rATP	tube with clear cap	tube with clear cap
HS Wash 1 Solution	bottle	bottle
HS Wash 2 Solution	bottle	bottle
HS Capture Solution	bottle	bottle
HS Elution Buffer	bottle	bottle
Primer 1	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	tube with blue cap
Herculase II Fusion DNA Polymerase	tube with clear cap	tube with clear cap
Herculase II Reaction Buffer	bottle	bottle
100 mM dNTP Mix	tube with clear cap	tube with clear cap
HaloPlex HS Probe	tube with pink cap	tube with pink cap
HaloPlex HS Indexing Primers	Indexing Primers A01 to H06 in orange 96-well plate†	Indexing Primers A01 to H12 in yellow 96-well plate‡

* re-Albumin Solution replaces the BSA Solution provided with earlier versions of this kit. Either reagent solution can be used in the protocols provided in this publication (see page 17 to page 18).

† See Table 15 for a plate map.

‡ See Table 16 for a plate map.

Table 15 Plate map for HaloPlex HS Indexing Primers A01 through H06 provided 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 16 Plate map for HaloPlex HS Indexing Primers A01 through H12 provided 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 HS Indexes

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex HS Indexing Primer is provided in the tables below. Yellow plates provided with 96-reaction kits contain all indexes A01 through H12. Orange plates provided with 48-reaction kits contain indexes A01 through H06.

Table 17 HaloPlex HS Indexes

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	TGACCGAT	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

In This Book

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

Version D0, February 2025

G9931-90000