

HaloPlex Target Enrichment System

For Illumina Sequencing

Protocol

Version G0, July 2018

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



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2015, 2018

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

G9900-90001

Edition

Version G0, July 2018

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Technical Support

For US and Canada

Call (800) 227-9770 (option 3,4,4)

Or send an e-mail to:

ngs.support@agilent.com

For Europe, Middle East, Africa, and India

Call 00800 345 600 (toll free) or
+49 69 8679 7730

Or send an e-mail to:

genomics_tech_europe@agilent.com

For all other regions

Agilent's world-wide sales and support center telephone numbers can be obtained at www.agilent.com/genomics under Contact Us.

Or send an e-mail to:

ngs.support@agilent.com

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A **WARNING** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a **WARNING** notice until the indicated conditions are fully understood and met.

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 G0

- Support for new Tier 1 Plus probe designs (see [Table 2](#) on page 10, [Table 7](#) on page 28, and [Table 12](#) on page 48)
- Removal of support information for discontinued kits p/n G9903A, G9903B, G9908A, G9908B, G9913A, G9913B ([Table 2](#) on page 10, [Table 13](#) on page 49 and [Table 14](#) on page 50)
- Updates to sequencing support details (see [page 42](#) through [page 45](#))
- Update to acceptable electrophoresis result description for restriction digest G (see *Note* on [page 22](#))
- Support for the Agilent 4200 TapeStation system (see [Table 4](#) on page 12 and see [page 24](#) and [page 40](#))
- Updates to Agilent 2100 Bioanalyzer system ordering information (see [Table 4](#) on page 12)
- Updates to supplier name for materials purchased from Thermo Fisher Scientific (see [Table 1](#) on page 9, [Table 3](#) on page 11, and [Table 4](#) on page 12)
- Removal of reference information for obsolete kits containing 8-bp indexing primers 1–96, provided in clear-capped tubes or clear plate (typically received before December 2014). If you need assistance with kits containing these obsolete indexing primer components, please contact [Technical Support](#).
- Updates to Technical Support contact information (see [page 2](#))

What's New in Version F1

- Support for ClearSeq Disease Research Panels (see [Table 2](#) on page 10 and [Table 13](#) on page 49)
- Updates to format of hybridization instructions (see [Table 7](#) on page 28)
- Update to name of indexing primers (see [Table 14](#) on page 50)

Content

1 Before You Begin

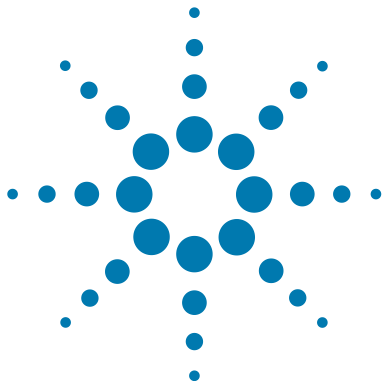
Procedural Notes	8
Safety Notes	8
Required Reagents	9
Required Equipment	11
Optional Validation Reagents and Equipment	12

2 Sample Preparation

Run Size Considerations	15
Run Time Considerations	15
Step 1. Digest genomic DNA with restriction enzymes	16
Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing	26
Step 3. Capture the target DNA	29
Step 4. Ligate the captured, circularized fragments	32
Step 5. Prepare the PCR Master Mix	33
Step 6. Elute captured DNA with NaOH	34
Step 7. PCR amplify the captured target libraries	35
Step 8. Purify the amplified target libraries	36
Step 9. Validate enrichment and quantify enriched target DNA	38
Step 10. Pool samples with different indexes for multiplexed sequencing	42

3 Reference

Kit Contents	48
Nucleotide Sequences of HaloPlex Indexes	52
Qualitative analysis of enrichment by gel electrophoresis	53



1 Before You Begin

Procedural Notes	8
Safety Notes	8
Required Reagents	9
Required Equipment	11
Optional Validation Reagents and Equipment	12

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

Description	Vendor and part number
HaloPlex Target Enrichment System Kit or ClearSeq 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)	Thermo Fisher Scientific p/n 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	Thermo Fisher Scientific p/n Q32850 Thermo Fisher Scientific 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 enrichment reactions).

1 Before You Begin

Required Reagents

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

Table 2 HaloPlex and ClearSeq Target Enrichment System Kits for Illumina Sequencing

Probe Design	Part Number	
	96 Reactions	48 Reactions
HaloPlex Custom Panel Tier 1 [*] , ILM	G9901B	G9901C
HaloPlex Custom Panel Tier 1 Plus [*] , ILM	G9961B	G9961C
HaloPlex Custom Panel Tier 2 [†] , ILM	G9911B	G9911C
HaloPlex Custom Panel Tier 3 [‡] , ILM	G9921B	G9921C
ClearSeq ICCG, ILM	G9953B ^{**}	G9953C ^{**}
ClearSeq Connective Disorder, ILM	G9953B ^{**}	G9953C ^{**}
ClearSeq Arrhythmia, ILM	G9953B ^{**}	G9953C ^{**}
ClearSeq Noonan Syndrome, ILM	G9953B ^{**}	G9953C ^{**}
ClearSeq Chromosome X, ILM	G9953B ^{**}	G9953C ^{**}

* Tier 1 designs are 1-500 kb with <15,000 probes and require a 3-hour hybridization protocol. Tier 1 Plus designs are also 1-500 kb, but contain 15,000 to 20,000 probes and require a 16-hour hybridization protocol.

† 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.

** Select the appropriate made-to-order probe option in SureDesign.

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 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, 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- μ L and 100- μ L 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

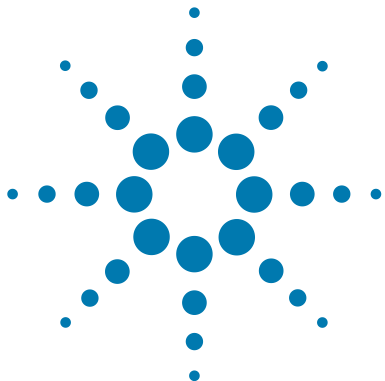
* Thermal cycler must have a maximum reaction volume specification of at least 100 μ L and must be compatible with 0.2 mL tubes.

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
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
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

* DNA samples may also be analyzed using the Agilent 2200 TapeStation, p/n G2964AA or G2965AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.



2 Sample Preparation

- Run Size Considerations 15
- Run Time Considerations 15
- Step 1. Digest genomic DNA with restriction enzymes 16
- Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing 26
- Step 3. Capture the target DNA 29
- Step 4. Ligate the captured, circularized fragments 32
- Step 5. Prepare the PCR Master Mix 33
- Step 6. Elute captured DNA with NaOH 34
- Step 7. PCR amplify the captured target libraries 35
- Step 8. Purify the amplified target libraries 36
- Step 9. Validate enrichment and quantify enriched target DNA 38
- Step 10. Pool samples with different indexes for multiplexed sequencing 42

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.



2 Sample Preparation

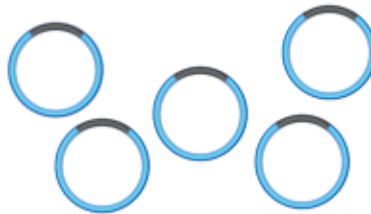
1) Digest genomic DNA.



2) Hybridize the HaloPlex probe library in presence of the Indexing Primer Cassette. Hybridization results in gDNA fragment circularization and incorporation of indexes and Illumina sequencing motifs.



3) Capture target DNA-probe hybrids. Biotinylation of probe DNA allows capture using streptavidin-coated magnetic beads.



4) PCR amplify targeted fragments to produce a sequencing-ready, target-enriched sample.



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

Run Size Considerations

Kits contain enough reagents for 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.

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 <15,000 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 $\geq 15,000$ probes use a 16-hour hybridization time, which is typically completed overnight, with the DNA digestion step started in the afternoon.

2 Sample Preparation

Step 1. Digest genomic DNA with restriction enzymes

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 μ L nuclease-free water, for a final DNA concentration of 5 ng/ μ L. Store on ice.
 - b In a separate 0.2-mL PCR tube, dispense 45 μ L 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.

Step 1. Digest genomic DNA with restriction enzymes

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](#).

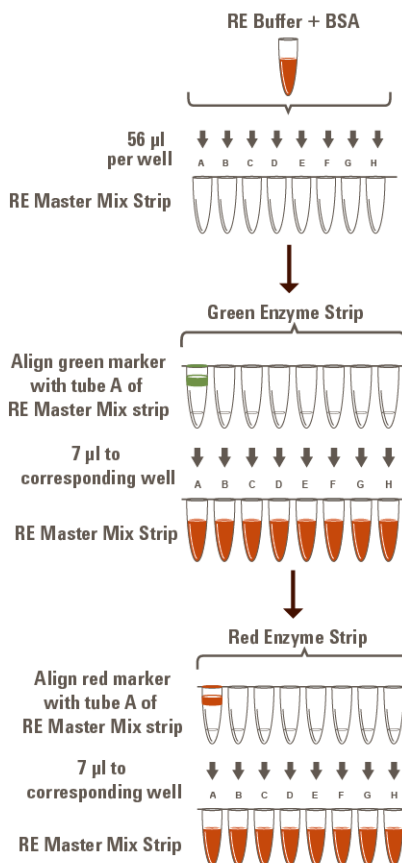


Figure 2 Preparation of the Restriction Enzyme Master Mix Strip for 12-sample run.

2 Sample Preparation

Step 1. Digest genomic DNA with restriction enzymes

- 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 μ L	476 μ L
BSA Solution	0.85 μ L	11.9 μ L
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 μ L	56 μ L

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 μ L	7 μ L

Step 1. Digest genomic DNA with restriction enzymes

- 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 μ L	7 μ L

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

For runs with >12 samples, continue distributing 5 μ L 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).

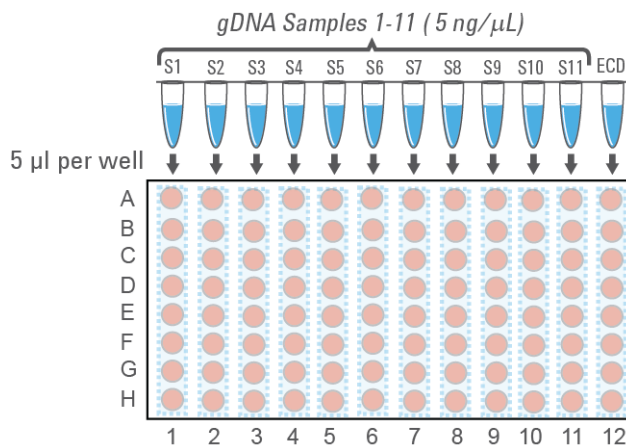


Each row of the 96-well plate now contains 5 μ L per well of the same restriction enzyme combination.

2 Sample Preparation

Step 1. Digest genomic DNA with restriction enzymes

- 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.

Step 1. Digest genomic DNA with restriction enzymes

- 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 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 26](#), upon which restriction enzymes are inactivated by the reaction conditions.

2 Sample Preparation

Step 1. Digest genomic DNA with restriction enzymes

- 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 μ L of each ECD digestion reaction from wells of the digestion reaction plate to fresh 0.2-mL PCR tubes.
- b Incubate the removed 4- μ L samples at 80°C for 5 minutes to inactivate the restriction enzymes.
- c Analyze the prepared samples by electrophoresis using the Agilent 2100 Bioanalyzer (see [page 23](#)), the Agilent 4200 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 reactions B and G to be slightly reduced, compared to the other digestion reactions.

Option 1: Validation by Agilent 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 *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com.

- 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.

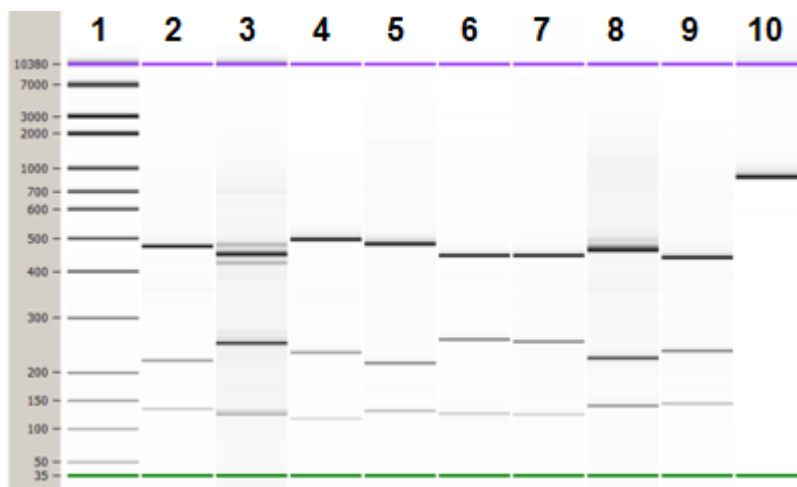


Figure 3 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

2 Sample Preparation

Step 1. Digest genomic DNA with restriction enzymes

Option 2: Validation by Agilent 4200 TapeStation analysis

Use a High Sensitivity D1000 ScreenTape and reagent kit. For more information to do this step, see the [Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System](#).

- 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 4200 TapeStation and start the run. See [Figure 4](#) for sample TapeStation electrophoresis results.

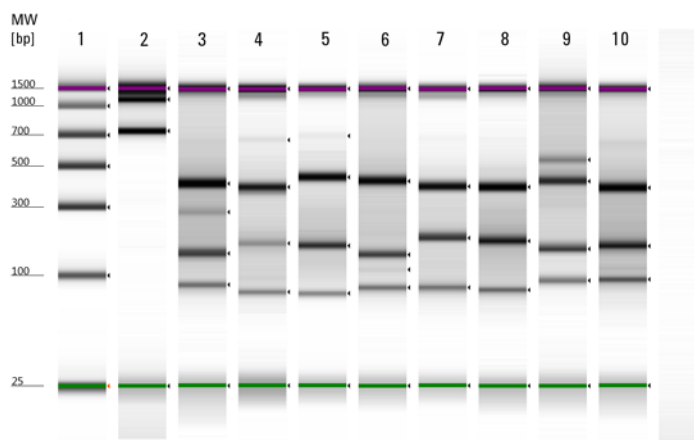


Figure 4 Validation of restriction digestion by TapeStation analysis. Lane 1: High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

Option 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.

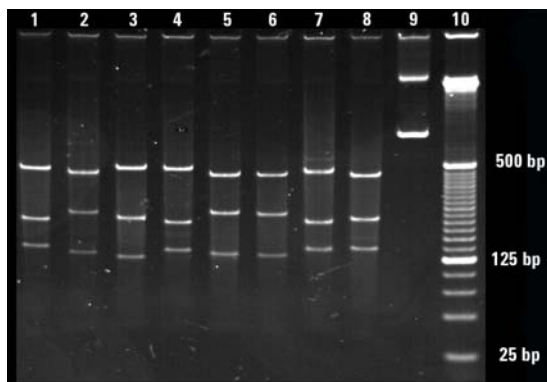


Figure 5 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

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 35](#).

2 Sample Preparation

Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing

Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex or ClearSeq 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 or ClearSeq 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 μ L	650 μ L
HaloPlex or ClearSeq Probe	20 μ L	260 μ L
Total Volume	70 μL	910 μL

- 2 Distribute 70 μ L of the Hybridization Master Mix to each of 12 0.2-mL tubes.
- 3 Add 10 μ L of the appropriate 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.
- 4 Transfer digested DNA samples from the 96-well Restriction Digest Reaction Plate(s) directly into the hybridization reaction tubes prepared in [step 3](#).

Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing

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
- 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.

2 Sample Preparation

Step 2. Hybridize digested DNA to HaloPlex or ClearSeq probe for target enrichment and sample indexing

- 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 probe hybridization

Step	Temperature	Time (Duration of Step)	
		Designs with <15,000 probes (see Certificate of Analysis)	Designs with ≥15,000 probes (see Certificate of Analysis)
Step 1	95°C	10 minutes	10 minutes
Step 2	54°C	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.

CAUTION

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

The 160-µL 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-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 $+4^{\circ}\text{C}$ 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 33](#).

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 34](#).
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.

2 Sample Preparation

Step 3. Capture the target DNA

- 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.

2 Sample Preparation

Step 4. Ligate the captured, circularized fragments

Step 4. Ligate the captured, circularized fragments

In this step, DNA ligase is added to the capture reaction to close nicks in the circularized 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 μ L	617.5 μ L
DNA Ligase	2.5 μ L	32.5 μ L
Total Volume	50 μL	650 μL

- 2 Add 50 μ L 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- μ L multichannel pipette set to 40 μ L.
- 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 35](#).

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 34](#).

- 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 35](#).

2 Sample Preparation

Step 6. Elute captured DNA with NaOH

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 libraries.

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 29](#).

- 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 μ L.
- 3 Remove the tubes from the magnetic plate and add 100 μ L of the SSC Buffer provided with the kit to each tube.
- 4 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100- μ L multichannel pipette set to 80 μ L.
- 5 Briefly spin the tubes and then return the tubes to the magnetic plate.
- 6 Wait for the solution to clear (about 30 seconds), then carefully remove and discard the SSC Buffer using a multichannel pipette set to 120 μ L. If necessary, carefully remove any residual liquid with a 20- μ L volume pipette.
- 7 Add 25 μ L of 50 mM NaOH, which was freshly-prepared on [page 29](#), to each tube.
- 8 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100- μ L multichannel pipette set to 15 μ L.
- 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 μ L of cleared supernatant from each tube on the magnetic plate to a PCR Master Mix tube held on ice (from [page 33](#)).
- 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 or ClearSeq Probe design. Consult the Certificate of Analysis (provided with Box 1 of your kit) for the PCR cycling recommendation for your probe.

Table 10 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.

2 Sample Preparation

Step 8. Purify the amplified target libraries

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.

2 Sample Preparation

Step 9. Validate enrichment and quantify enriched target DNA

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 using the Agilent 2100 Bioanalyzer (see [page 39](#)) or 4200 TapeStation (see [page 40](#)).

Enriched library samples may also be qualitatively analyzed using gel electrophoresis. Sample gel electrophoresis results are provided in the [Reference](#) section on [page 53](#).

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 target-enriched 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.

Step 9. Validate enrichment and quantify enriched target DNA

Option 1: Analysis using the Agilent 2100 Bioanalyzer

Use a Bioanalyzer High Sensitivity DNA Assay kit and the Agilent 2100 Bioanalyzer with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

- 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 instrument and start the run within five minutes after preparation.
- 3 Analyze the electropherogram for each sample using the analysis guidelines on [page 41](#).

See [Figure 7](#) for a sample electropherogram.

NOTE

If the concentration determined by Bioanalyzer analysis is $> 10 \text{ ng}/\mu\text{L}$, 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.

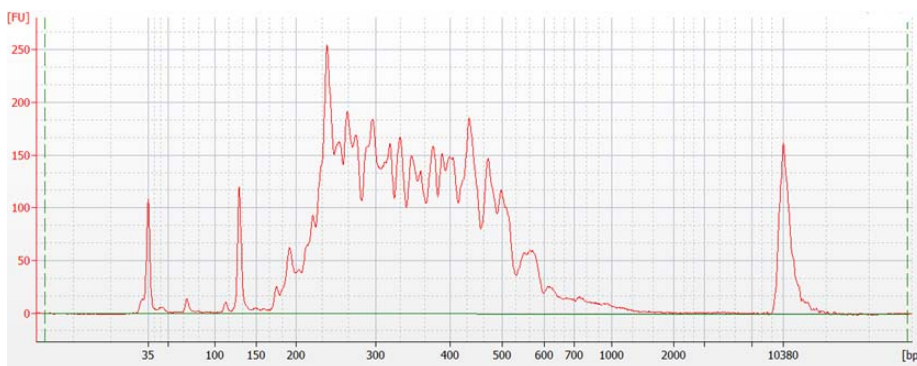


Figure 7 Validation of enrichment by Agilent 2100 Bioanalyzer analysis.

2 Sample Preparation

Step 9. Validate enrichment and quantify enriched target DNA

Option 2: Analysis using the Agilent 4200 TapeStation

Use a High Sensitivity D1000 ScreenTape and reagent kit. For more information to do this step, see the [Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System](#).

- 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 41](#).

See [Figure 8](#) for a sample electropherogram.

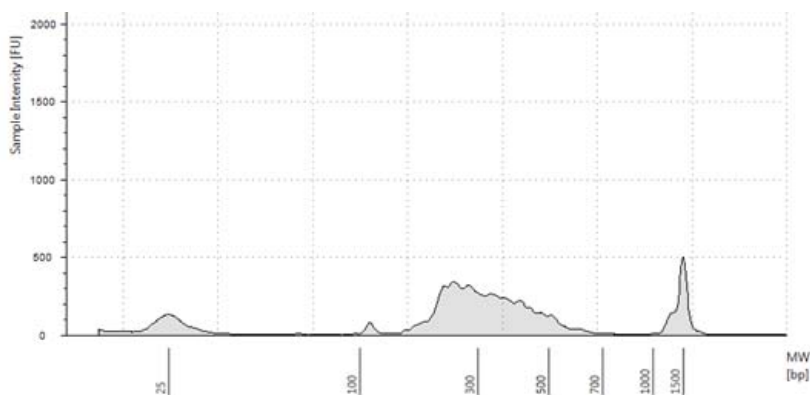


Figure 8 Validation of enrichment by TapeStation analysis.

Step 9. Validate enrichment and quantify enriched target DNA

Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 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 μ L of the pooled libraries, purify the DNA using AMPure XP beads according to the protocol on [page 36](#).

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 or MiSeq platform. See additional guidelines for the MiSeq platform (below) and HiSeq platform ([page 45](#)).
- 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 47](#).
- 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 47](#), 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*.

Step 10. Pool samples with different indexes for multiplexed sequencing

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

Illumina Experiment Manager

Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings

Reagent Cartridge Barcode* MS5871368-300V2

Library Prep Workflow TruSeq Nano DNA

Index Adapters TruSeq DNA Single Indexes (A,B)

Index Reads 0 (None) 1 (Single) 2 (Dual)

Experiment Name

Investigator Name

Description

Date 1/22/2018

Read Type Paired End Single Read

Cycles Read 1 100

Cycles Read 2 100

* - required field

FASTQ Only Workflow-Specific Settings

Custom Primer for Read 1

Custom Primer for Index

Custom Primer for Read 2

Reverse Complement

Use Adapter Trimming

Use Adapter Trimming Read 2

2 Sample Preparation

Step 10. Pool samples with different indexes for multiplexed sequencing

- 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 table, assign each sample to any of the Illumina 17 indexes. The index will be corrected to a HaloPlex index at a later stage.

	Sample ID*	Sample Name	Index 1 (17)*	Sample Project	Description
A01	1	Sample1	A001	ProjectX	Tumor
A02	2	Sample2	A002	ProjectX	Normal
A03	3	Sample3	A003	ProjectX	Tumor

- Finish the sample plate setup tasks and save the sample plate file.
- 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:

- 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.

```
17 [Settings]
18
19 [Data]
20 Sample_ID,Sample_Name,Sample_Plate,Sample_Well,I7_Index_ID,index,Sample_Project,Description
21 1,Sample1,testplate,A01,A001,ATCACG,ProjectX,Tumor
22 2,Sample2,testplate,A02,A002,CGAAGT,ProjectX,Normal
23 3,Sample3,testplate,A03,A003,TTAGGC,ProjectX,Tumor
24 4,Sample4,testplate,A04,A004,TGACCA,ProjectX,Normal
25 5,Sample5,testplate,A05,A005,ACAGTG,ProjectX,Tumor
26 6,Sample6,testplate,A06,A006,AGT...
```

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

Step 10. Pool samples with different indexes for multiplexed sequencing

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

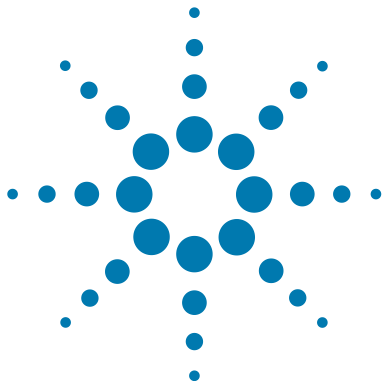
Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	0
Read 2	100

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.

2 **Sample Preparation**

Step 10. Pool samples with different indexes for multiplexed sequencing



3 Reference

Kit Contents 48
Nucleotide Sequences of HaloPlex Indexes 52
Qualitative analysis of enrichment by gel electrophoresis 53

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



Kit Contents

HaloPlex Target Enrichment System Kits for custom designs include the component kits listed in [Table 12](#) below. Kits for ClearSeq Disease Research Panels include the component kits listed in [Table 13](#) below.

Table 12 HaloPlex Target Enrichment System Kit Contents-Custom Designs

Design Type	Reaction Number	HaloPlex Target Enrichment System-ILM, Box 1 [†]	HaloPlex Magnetic Beads Box 2
		Store at -20°C	Store at +4°C
HaloPlex 1-500 kb with <15,000 Probes, ILM	48 Reactions	5190-8044 OR 5190-8045 ^{††}	5190-5976
	96 Reactions	5190-8050 OR 5190-8051 [†]	5190-5386
HaloPlex 1-500 kb with 15,000-20,000 Probes, ILM	48 Reactions	5191-4066 OR 5191-4067 [†]	5190-5976
	96 Reactions	5191-4068 OR 5191-4069 [†]	5190-5386
HaloPlex 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
HaloPlex 2.6 Mb-5 Mb, ILM	48 Reactions	5190-8048 OR 5190-8049 [†]	5190-5976
	96 Reactions	5190-8054 OR 5190-8055 [†]	5190-5386

* See [Table 14](#) for list of included reagents.

† Part number 5190-8044, 5190-8050, 5191-4066, 5191-4068, 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, 5191-4067, 5191-4069, 5190-8047, 5190-8053, 5190-8049 or 5190-8055.

Table 13 HaloPlex Target Enrichment System Kit Contents-ClearSeq Disease Research Panels

Design Type	Reaction Number	HaloPlex Target Enrichment System-ILM, Box 1	HaloPlex Magnetic Beads Box 2
		Store at -20°C	Store at +4°C
ClearSeq ICCG, ILM	48 Reactions	5190-9146	5190-9223
	96 Reactions	5190-9164	5190-9224
ClearSeq Connective Disorder, ILM	48 Reactions	5190-9150	5190-9223
	96 Reactions	5190-9168	5190-9224
ClearSeq Arrhythmia, ILM	48 Reactions	5190-9144	5190-9223
	96 Reactions	5190-9162	5190-9224
ClearSeq Noonan Syndrome, ILM	48 Reactions	5190-9152	5190-9223
	96 Reactions	5190-9170	5190-9224
ClearSeq Chromosome-X, ILM	48 Reactions	5190-9148	5190-9223
	96 Reactions	5190-9166	5190-9224

* See [Table 14](#) for list of included reagents.

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

Table 14 HaloPlex Target Enrichment System Box 1 Contents

Included Reagents	48 Reaction Kit	96 Reaction Kit
Hybridization Solution	bottle	bottle
Ligation Solution	bottle	bottle
Wash Solution	bottle	bottle
Capture Solution	bottle	bottle
SSC Buffer	bottle	bottle
RE Buffer	bottle	bottle
BSA Solution	tube with clear cap	tube with clear cap
DNA Ligase	tube with red cap	tube with red cap
Enrichment Control DNA	tube with orange cap	tube with orange cap
Primer 1	tube with yellow cap	tube with yellow cap
Primer 2	tube with blue cap	tube with blue cap
HaloPlex Indexing Primers	96-well plate with Indexing Primer A01 to H06 (blue plate)*	96-well plate with Indexing Primer A01 to H12 (blue plate)†
Enzyme Strip 1	8-well strip tube with green label	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label
HaloPlex or ClearSeq Probe	tube with pink cap	tube with pink cap

* See [Table 15](#) for a plate map.

† See [Table 16](#) for a plate map.

Table 15 Plate map for HaloPlex 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 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

3 Reference

Nucleotide Sequences of HaloPlex Indexes

Nucleotide Sequences of HaloPlex Indexes

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex Indexing Primer is provided in the table below.

Table 17 HaloPlex Indexes, for indexing primers provided in blue 96-well plate

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

Qualitative analysis of enrichment by gel electrophoresis

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

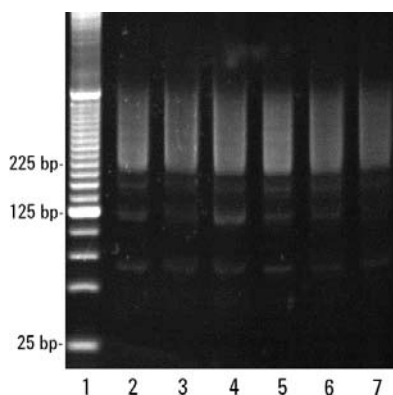


Figure 9 Validation of HaloPlex enrichment process by gel electrophoresis. Lane 1: 25-bp DNA ladder, Lanes 2-7: enriched library samples.

www.agilent.com

In This Book

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

© Agilent Technologies, Inc. 2015, 2018

Version G0, July 2018



G9900-90001



Agilent Technologies