HaloPlex HS Target Enrichment System

Automation Protocol

For Illumina Sequencing

Protocol

Version D0, February 2025

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

Agilent Technologies

Notices

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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 all other regions

Agilent's world-wide Sales and Support Center contact details for your location can be obtained at
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In this Guide...

This guide describes an optimized automation protocol for using the HaloPlex HS target enrichment system to prepare sequencing library samples for Illumina paired-end multiplexed sequencing platforms. Sample processing steps are automated using the Agilent NGS Bravo Option A.

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 Using the Agilent NGS Bravo for HaloPlex HS Target Enrichment

This chapter contains an orientation to the Agilent NGS Bravo Option A, an overview of the HaloPlex HS target enrichment protocol, and considerations for designing HaloPlex HS experiments for automated processing using the Agilent NGS Bravo Option A.

3 Sample Preparation

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

4 Reference

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

5 Appendix I: Using FFPE-derived DNA Samples

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

6 Appendix II: Provisional Adaptor-Dimer Removal Protocol

This chapter describes a protocol used to remove adaptor-dimers that may be observed for some designs.

What's New in Version D0

- Support for kits supplied with re-Albumin Solution, replacing BSA Solution (see page 33, page 34, and page 78). The protocols in this publication are compatible with use of either form of albumin-containing solution.
- Updates to Technical Support contact information on page 2
- Updates to Notices on page 2
- New *Procedural Note* on use of compression pads where applicable (see page 8)
- Updates to required materials supplier information in Table 1 on page 9 and Table 3 on page 11, including removal of the SureCycler thermal cycler recommendation
- Updates to information on use of Agilent's SureDesign for access to HaloPlex probe designs and kit ordering information on page 10, page 30 and page 81
- Updates to lists of available HaloPlex HS kits in Table 2 on page 10 and Table 35 on page 77
- Updates to NGS Bravo system information in Table 3 on page 11 and on page 15 and page 19
- Updates to purchasing information for the Agilent 2100 Bioanalyzer instrument in Table 4 on page 12, and related updates to validation instructions on page 41 to page 42 and page 71 to page 72
- Updates to Agilent.com webpage hyperlinks
- Updates to downstream sequencing support information on page 74 and page 75
- 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 96 reaction kit contains enough reagents to prepare master mixes for four runs of 3 columns of samples (24 samples) per run. When processing samples using runs with fewer than 24 samples, some reagents may be depleted before 96 samples are run.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- 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.
- 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 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
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 Automated Protocols

Description	Vendor and part number
Agilent NGS Bravo Option A*	Contact Agilent Automation Solutions for ordering and VWorks software version information: Customerservice.automation@agilent.com
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402#226
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal Cycler	Various suppliers† *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401 or 951020619 • 384 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020702
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
Magnetic separator ‡ 1.5 mL tube-compatible separator or Conical vial-compatible separator	Thermo Fisher Scientific DynaMag-2 magnet, p/n 12321D or equivalent DynaMag-15 magnet, p/n 12301D 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
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Qubit 2.0 Fluorometer	Thermo Fisher Scientific p/n Q32866
Qubit assay tubes	Thermo Fisher Scientific p/n Q32856
Vortex mixer	General laboratory supplier
Decontamination wipes	General laboratory supplier

* Protocols are also compatible with Agilent NGS Workstation Option B. See page 13 for more information.

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

‡ Select the appropriate device based on run size. See page 56 to determine magnetic bead volume to be used for your run size.

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.

Using the Agilent NGS Workstation Option B for HaloPlex HS Automation

The detailed protocols in the following chapters are for the Agilent NGS Workstation Option A, but HaloPlex HS target enrichment protocols are also compatible with the Agilent NGS Workstation Option B. Depending on the configuration of the system purchased, however, additional adapters may be required. Before initiating experiments, see Table 5 below, and erify that the listed adapters are available for your workstation.

Table 5 Adapter checklist for HaloPlex automation using Agilent NGS Workstation Option B

Adapter Description	Quantity Required for HaloPlex Automation	Agilent part number (single adapter)
384-well plate insert	2	G5498B#60
96-well PCR plate insert (red)*	2	G5498B#13

* If your NGS Workstation Option B system is already equipped with one red insert, purchase one additional insert using the ordering information shown.

2 Using the Agilent NGS Bravo for HaloPlex HS Target Enrichment

This chapter contains an orientation to the Agilent NGS Bravo (Option A), an overview of the HaloPlex HS target enrichment protocol, and considerations for designing HaloPlex HS experiments for automated processing using the Agilent NGS Bravo.

About the Agilent NGS Bravo Option A

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices and software. Refer to the support resources listed in Table 6.

Review the automation device support resources using the links provided in Table 6 to become familiar with the general features and operation of the automation components. Instructions in this user guide detail use of these automation components specifically for the HaloPlex HS Target Enrichment workflow.

Table 6 Agilent NGS Bravo User Guide reference information

Device	Link
Bravo Platform	Bravo Platform User Guide
VWorks Software	VWorks Automation Control Software Support
PlateLoc Thermal Microplate Sealer	PlateLoc Thermal Microplate Sealer Device Support

About the Bravo Platform

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck, suitable for handling 96-well, 384-well, and 1536-well plates. The Bravo platform is controlled by the VWorks Automation Control software. Fitted with a choice of three interchangeable fixed-tip or disposable-tip pipette heads, it accurately dispenses fluids from 0.3 µL to 250 µL.

Bravo Platform Deck

The protocols in the following sections include instructions for placing plates and reagent reservoirs on specific Bravo deck locations. Use Figure 1 to familiarize yourself with the location numbering convention on the Bravo platform deck.

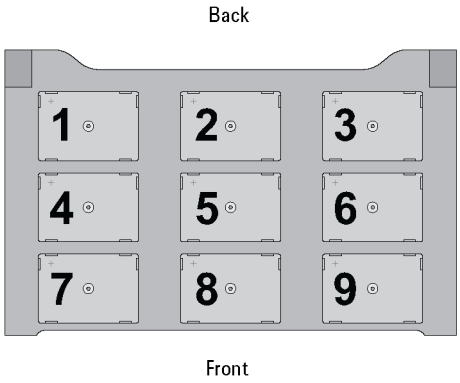


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

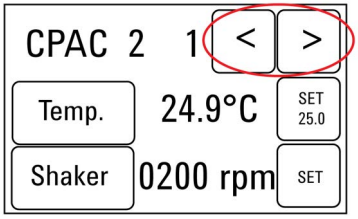
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include low-temperature (4°C) or high-temperature (54°C) incubation steps may be expedited by pre-setting the temperature of the affected block before starting the run.

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See Table 7 for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

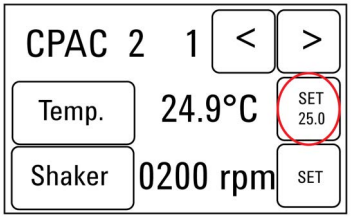
Table 7 Inheco Multi TEC Control touchscreen designations

Bravo Deck Position	Designation on Inheco Multi TEC Control Screen
4	CPAC 2 1
6	CPAC 2 2

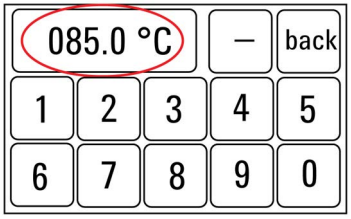
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



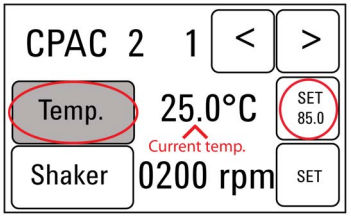
2 To set the temperature of the selected block, press the SET button.



3 Using the numeral pad, enter the desired temperature. The entered temperature appears in the top, left rectangle. Once the correct temperature is displayed, press the rectangle to enter the temperature.



4 Press the Temp button until the new temperature is displayed on the SET button and until the Temp button is darkened, indicating that the selected heat block is heating or cooling to the new temperature setting. The current temperature of the block is indicated in the center of the display.



VWorks Automation Control Software

VWorks software, included with your Agilent NGS Bravo, allows you to control the robot and integrated devices using a PC. The Agilent NGS Bravo Option A is preloaded with VWorks software containing all of the necessary HaloPlex HS system liquid handling protocols. General instructions for starting up the VWorks software and the included protocols are provided below. Each time a specific VWorks protocol is used in the HaloPlex HS procedure, any settings required for that protocol are included in the relevant section of this manual.

NOTE If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

1 Double-click the VWorks icon or the HaloPlex_HS.VWForm shortcut on the Windows desktop to start the VWorks software.

2 If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.

3 In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

Using the HaloPlex_HS.VWForm to setup and start a run

Use the VWorks form HaloPlex_HS.VWForm, shown below, to set up and start each HaloPlex HS automation protocol.

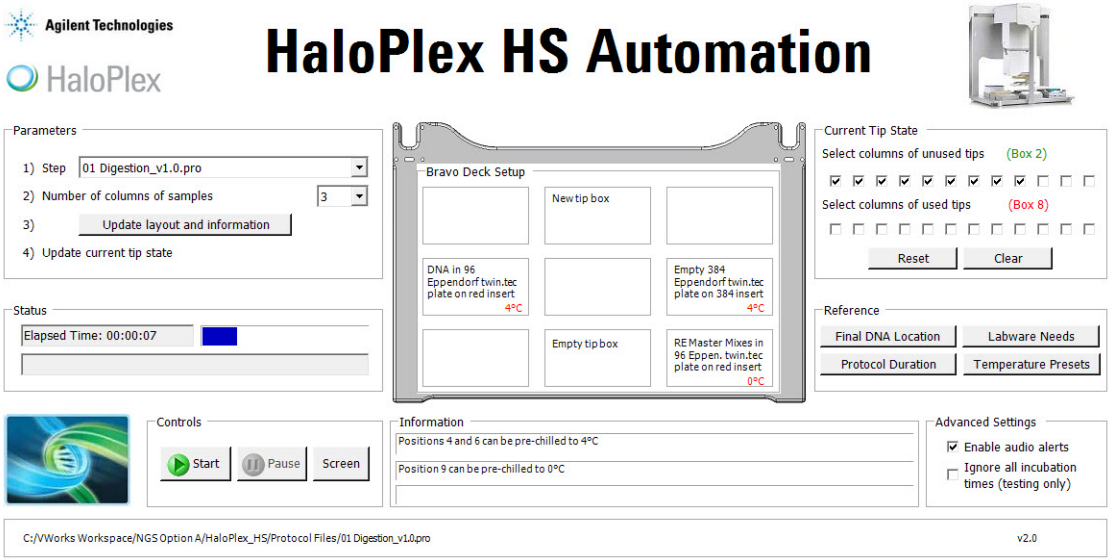
1 Open the form using the shortcut on your desktop.

2 Use the drop-down menus on the form to select the appropriate HaloPlex HS workflow step and number of columns of samples for the run.

3 Once all run parameters have been specified on the form, click **Update layout and information.**

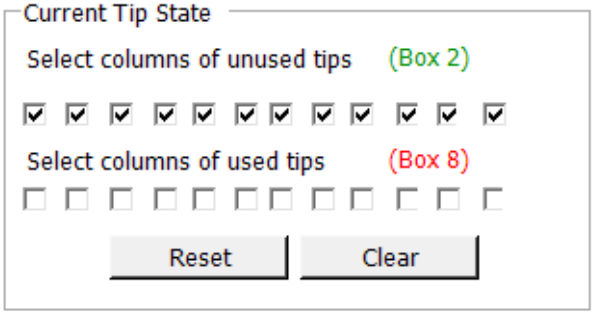
NOTE The displayed protocol will not run unless the **Update layout and information** button has been clicked.

4 The **Bravo Deck Setup** region of the form will then display the required placement of reaction components and labware on the NGS Bravo deck for the specified run parameters.



5 Verify that the **Current Tip State** indicator on the form (shown below) matches the configuration of unused tips in the tip box at Bravo Deck position 2.

For a fresh tip box, containing 12 columns of tips, all positions of the **Current Tip State** unused tip indicator (top portion, Box 2) should be selected, as shown below. Clicking **Reset** selects all columns for position 2.



Also verify that the used tip indicator (bottom portion, Box 8) matches the configuration of used tips in the tip box at Bravo Deck position 8.

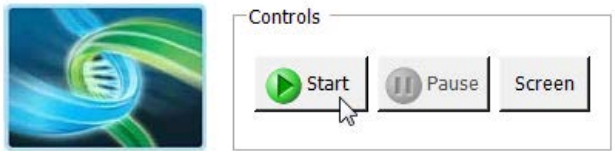
For an empty tip box, all positions of the **Current Tip State** used tip indicator (bottom portion, Box 8) should be cleared, as shown above. Clicking **Reset** clears all columns for position 8.

NOTE

It is important that the **Current Tip State** indicator matches the configuration of tips present at Bravo Deck positions 2 and 8 when initiating the run. Tips that are inappropriately loaded onto the Bravo platform pipette head, or tips missing from the pipette head, will interfere with automated processing steps.

You can use partial tip boxes for HaloPlex HS automation protocols, as long as positions of available tips are accurately indicated during run setup.

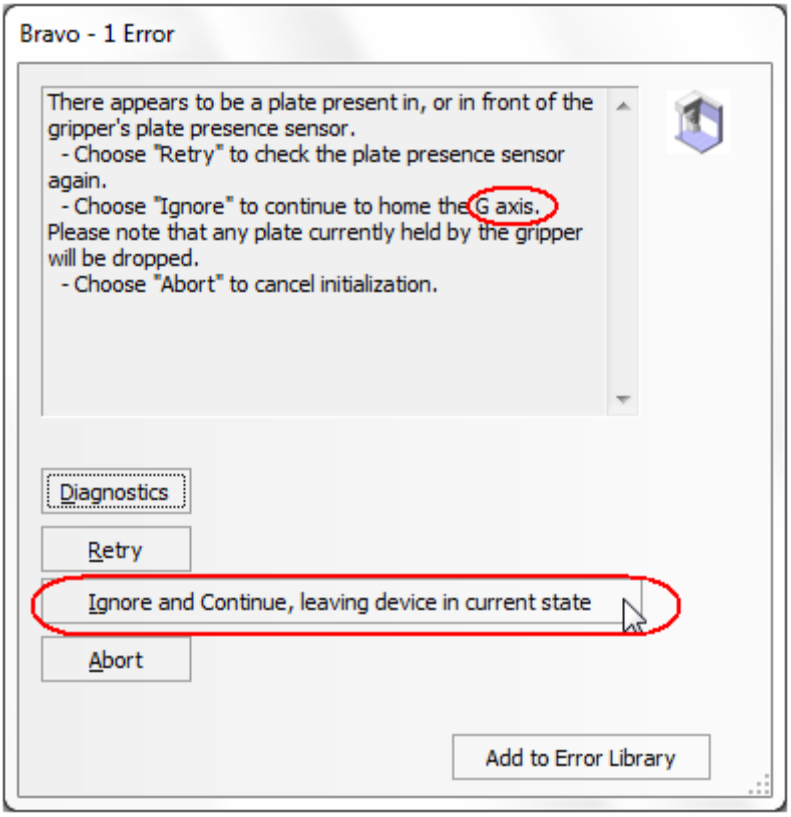
6 After verifying that the NGS Bravo has been set up correctly, click **Start** in the **Controls** section of the form to begin the run. Do not use the Start button on the VWorks Control Toolbar; runs must be initiated using the start button on the HaloPlex_HS.VWForm, shown below.



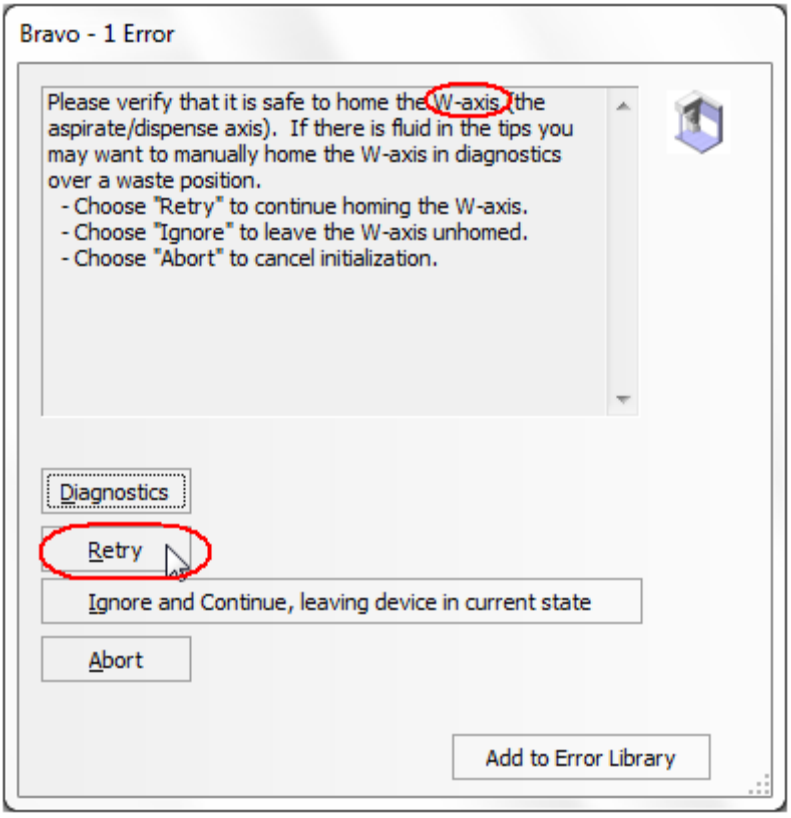
Error messages encountered at start of run

After starting the run, you may see the error messages displayed below. When encountered, make the indicated selections and proceed with the run. Encountering either or both of these error messages is not indicative of a problem with the NGS Bravo or your run setup.

1 If you encounter the G-axis error message shown below, select **Ignore and Continue, leaving device in current state**.



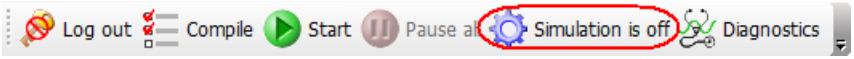
2 If you encounter the W-axis error message shown below, select **Retry.**



Verifying the Simulation setting

VWorks software may be run in simulation mode, during which commands entered on screen are not completed by the NGS Bravo. If NGS Bravo devices do not respond when you start a run, verify the simulation mode status in VWorks using the following steps.

1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View** > **Control Toolbar**).



2 If the indicator displays **Simulation is on,** click the status indicator button to turn off the simulation mode.

NOTE If you cannot see the toolbar above the HaloPlex HS. VWorks form, click **Screen** in the **Controls** section of the form to exit full screen mode. If the toolbar is still not visible, right-click on the form and then select **Control Toolbar** from the menu.

Overview of the HaloPlex HS Target Enrichment Procedure

Figure 2 summarizes the HaloPlex HS target enrichment workflow. For each sample to be sequenced, individual HaloPlex HS-enriched, indexed libraries are prepared. Depending on the specific sequencing platform used, up to 96 samples can be pooled and sequenced in a single lane.

Table 8 summarizes how the VWorks automation protocols are integrated into the HaloPlex HS workflow. See the Sample Preparation chapter for complete instructions for use of the VWorks protocols for sample processing.

Table 8 Overview of VWorks protocols used during the workflow

Workflow Step	VWorks Protocol used for Automation
Digest genomic DNA	Digestion_v1.0.pro
Hybridize to HaloPlex HS probe and index the samples	Hybridization_v1.0.pro
Close nicks in probe-target hybrids	Hyb_Purification_&_Ligation_v1.0.pro
Capture and wash enriched DNA	Capture_&_Wash_v1.0.pro
PCR-amplify the libraries	Amplification_v1.0.pro
Purify amplified libraries	Final_Purification_v1.0.pro

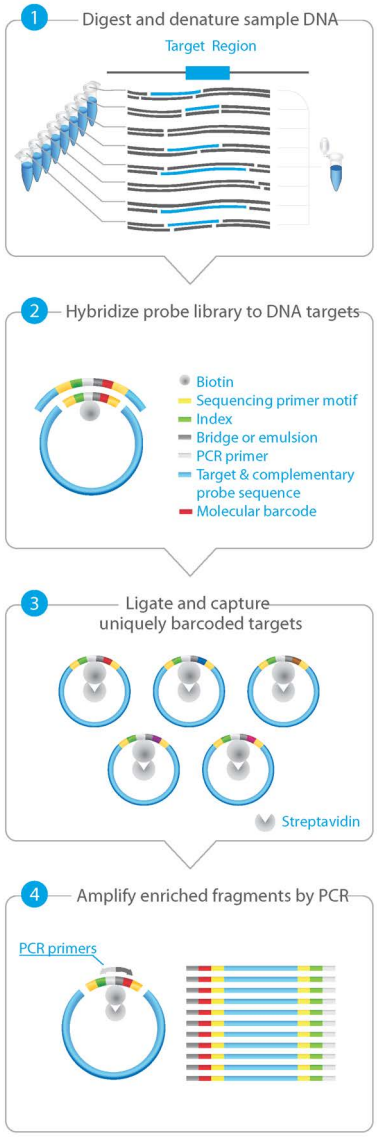


Figure 2 Overall HaloPlex HS target-enriched sequencing sample preparation workflow.

Experimental Setup Considerations for Automated Runs

HaloPlex HS Automated Target Enrichment System runs may include 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells) of gDNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Table 9 Columns to Samples Equivalency

Number of Columns Processed	Total Number of Samples Processed
1	8
2	16
3	24
4	32
6	48
12	96

The number of columns or samples that may be processed using the supplied reagents (see page 77) will depend on the experimental design. For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 96-reaction kit contains sufficient reagents for 96 reactions configured as 4 runs of 3 columns of samples per run.

Considerations for Placement of gDNA Samples in 96-well Plates for Automated Processing

- The Agilent NGS Bravo processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- For sample indexing during hybridization to the HaloPlex HS probe (see Figure 2), you will need to prepare a separate plate containing the HaloPlex HS Indexing Primers. Assign the wells to be indexed with their respective indexing primers during experimental design. See the Reference chapter for nucleotide sequences of the 96 indexes used in the HaloPlex HS Target Enrichment System.

Considerations for Equipment Setup

- Some workflow steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Bravo to allow rapid and efficient plate transfer.
- Some workflow steps require that the sample plate be sealed, then centrifuged to collect any dispersed liquid, before being transfered between instruments. To maximize efficiency, locate the PlateLoc thermal microplate sealer and the centrifuge in close proximity to the NGS Bravo and thermal cycler.

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. For these designs, DNA digestion through PCR protocols (see Figure 2) are typically run on the same day with the DNA digestion protocol initiated early in the day.

Designs containing >20,000 probes use a 16-hour hybridization time, which is typically completed overnight. Calculate the appropriate start time for the DNA digestion protocol, based on your run size and the run time estimates provided in the HaloPlex HS form in the VWorks software (HaloPlex_HS.VWForm), to allow overnight hybridization.

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 81 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).

3 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.

Step 1. Digest genomic DNA with restriction enzymes

In this step, gDNA samples are digested by 16 different restriction enzymes to create a library of gDNA restriction fragments. The gDNA is digested in eight different restriction reactions, each containing two restriction enzymes. **The 16 restriction enzymes are provided in two 8-well strip tubes that are distinguished by red and green color markers.** Enzymes are combined from corresponding wells of the red- and green-marked strip tubes to make eight different RE Master Mixes, which are then combined with each DNA sample in the run. 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 3 on page 32).

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a decontamination wipe.
2. Place red aluminum inserts on Bravo deck positions 4 and 9.
3. Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 ml of 25% ethanol.
4. Place a 384-well adapter insert on Bravo deck position 6.
5. Pre-set the temperature of Bravo deck positions 4 and 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.

NOTE

To expedite thermal cycler warm-up for the restriction digest incubation on page 38, you can enter and initiate the digestion program on the thermal cycler now, and then pause the program until you are instructed to transfer the reaction plate. Be sure to pause the thermal cycler before the initiation of the 37°C incubation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 14 on page 38. Be sure that the 384-well block is in the thermal cycler before initiating the program for warm-up.

Prepare the DNA Sample Source Plate

1. Use the Qubit dsDNA BR Assay or PicoGreen staining kit to determine the concentration of your gDNA samples.

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

NOTE

For FFPE-derived samples, use the guidelines on page 82 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, prepare a 3.6 ng/µl dilution of the sample DNA at this step, then use 32 µl in step 3 below.

3 Prepare the DNA sample plate for the run, containing up to 95 gDNA samples and the Enrichment Control DNA sample, using a full-skirted 96-well Eppendorf twin.tec plate.

NOTE

HaloPlex HS Automated Target Enrichment System runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. Use full columns of DNA samples for each run.

a In well A1 of a 96-well twin.tec plate, dispense 32 µl of the supplied Enrichment Control DNA (ECD). Store on ice.

b In separate wells of the same 96-well twin.tec plate, dispense 32 µl of each gDNA sample (1.8 ng/µl) to be included in the run.

For automated processing, fill plate wells column-wise in well order A1 to H1, then A2 to H2, ending with A12 to H12. Continue to store on ice.

Prepare the RE Master Mixes

1 Prepare the appropriate amount of RE Buffer + Albumin mixture, according to the table below.

Table 10 Preparation of RE Buffer + Albumin mixture for Digestion_v1.0.pro protocol

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
RE Buffer	24.5 µl	294 µl	490 µl	686 µl	882 µl	1274 µl	2548 µl
re-Albumin Solution*	0.5 µl	6 µl	10 µl	14 µl	18 µl	26 µl	52 µl
Total Volume	**25 µl**	**300 µl**	**500 µl**	**700 µl**	**900 µl**	**1300 µl**	**2600 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.

2 Obtain the two provided green- and red-marked Enzyme Strips from the HaloPlex HS kit. For each strip, label the color-marked tube with A, then continue labeling the remaining tubes with B through H, in order. Keep the strips on ice.

CAUTION It is important to use the restriction enzyme tube strips 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 are used to mark well A of each enzyme strip.

3 In eight individual tubes, prepare the eight Restriction Enzyme Master Mixes A, B, C, D, E, F, G, and H according to the table below. To prepare Master Mix A, combine RE Buffer + Albumin from step 1 with the indicated volumes of enzyme solution from well A of the Green Enzyme Strip and from well A of the Red Enzyme Strip. Prepare Master Mixes B–H by repeating this process using enzyme solutions from the corresponding wells B-H of each provided Enzyme Strip.

Table 11 Preparation of RE Master Mixes A–H for Digestion_v1.0.pro protocol

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
RE Buffer + Albumin	2.8 µl	35.7 µl	59.5 µl	83.3 µl	107.1µl	154.7 µl	309.4 µl
Green Enzyme Strip enzyme A–H	0.35 µl	4.5 µl	7.4 µl	10.4 µl	13.4 µl	19.3 µl	38.7 µl
Red Enzyme Strip enzyme A–H	0.35 µl	4.5 µl	7.4 µl	10.4 µl	13.4 µl	19.3 µl	38.7 µl
Total Volume for each Master Mix A, B, C, D, E, F, G, or H	**3.5 µl**	**44.7 µl**	**74.3 µl**	**104.1 µl**	**133.9 µl**	**193.3 µl**	**386.8 µl**

NOTE For 1-6 column runs, RE master mixes A-H may be prepared in a 8 x 0.2-ml well strip tube, using a multichannel pipette to transfer volumes from Enzyme Strips 1 and 2 to the RE master mix strip. For 12-column runs, prepare the master mixes in 1.5-ml tubes.

4 Mix by gentle vortexing and then spin briefly. Keep on ice.

Prepare the RE master mix source plate

1 Aliquot the Restriction Enzyme Master Mixes to a full-skirted 96-well Eppendorf twin.tec plate as shown in Figure 3. Add the volumes indicated in Table 12 of each master mix A–H to each well of the indicated column of the twin.tec plate. Keep the master mixes on ice during the aliquoting steps.

Table 12 Preparation of the RE Master Mix Source Plate for Digestion_v1.0.pro

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
RE Master Mix A	Column 1 (A1-H1)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix B	Column 2 (A2-H2)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix C	Column 3 (A3-H3)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix D	Column 4 (A4-H4)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix E	Column 5 (A5-H5)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix F	Column 6 (A6-H6)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix G	Column 7 (A7-H7)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl
RE Master Mix H	Column 8 (A8-H8)	5.1 µl	8.9 µl	12.6 µl	16.3 µl	23.7 µl	47.9 µl

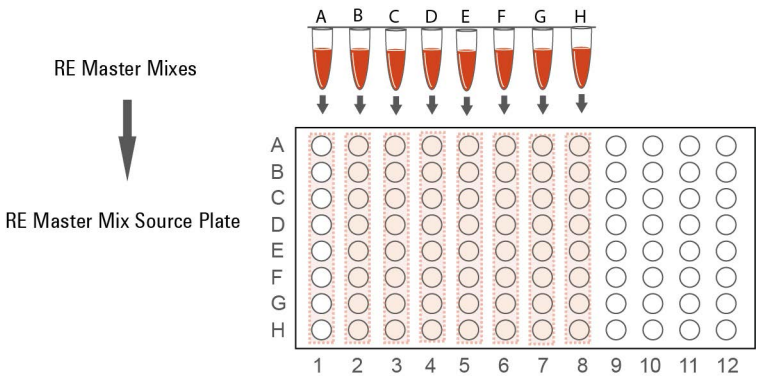


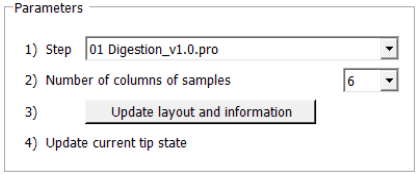
Figure 3 Preparation of the RE Master Mix source plate for automation protocol Digestion_v1.0.pro.

Load the NGS Bravo and Run the Digestion_v1.0.pro VWorks Protocol

1 Open the HaloPlex HS setup form using the HaloPlex_HS.VWForm shortcut on your desktop.

2 Log in to the VWorks software.

3 On the setup form, under **Step**, select **01 Digestion_v1.0.pro.**



4 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

5 Click **Update layout and information.**

6 Load the Bravo deck according to Table 13.

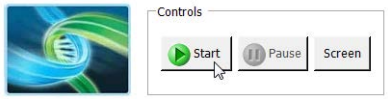
Table 13 Initial Bravo deck configuration for Digestion_v1.0.pro

Location	Content
1	—(empty)—
2	New tip box
3	For 12-column runs only: Empty 384-well Eppendorf twin.tec plate (no 384-well insert required) For 1- to 6-column runs: empty
4	gDNA samples in full-skirted 96-well Eppendorf twin.tec plate seated on red insert
5	—(empty)—
6	Empty 384-well Eppendorf twin.tec plate seated on 384-well insert
7	—(empty)—
8	Empty tip box
9	RE Master Mix source plate (full-skirted 96-well Eppendorf twin.tec plate) seated on red insert

7 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** and **Information** regions of the form.

8 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

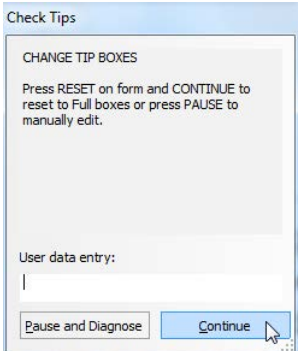
9 When verification is complete, click **Start** to start the run.



NOTE If Bravo devices do not respond when you start the run, but activity is recorded in the Log, verify that VWorks is not running in Simulation mode. See page 24 for more information.

10 When prompted by VWorks as shown below, replace the tip box at position 2 with a new tip box and replace the used tip box at position 8 with an empty tip box. After both tip boxes are in place, click **Reset** under **Current Tip State** on the form. Verify that the tip state was updated and then click **Continue** on the prompt shown below.

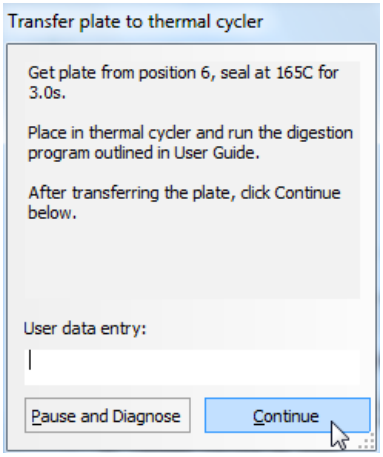
Depending on the run size, you may be prompted to change tip boxes multiple times during the run.



The NGS Bravo combines each gDNA sample with each RE Master Mix in wells of a 384-well reaction plate. For 1- to 6-column runs, a single 384-well restriction digest plate is prepared; for 12 column runs, two 384-well restriction digest plates are prepared.

11 When the NGS Bravo has finished preparing each 384-well restriction digest plate for the run, you will be prompted by VWorks as shown below.

The final Bravo deck position of the prepared restriction digest plate varies for different run sizes.



12 Remove the 384-well plate from the Bravo deck position indicated in the prompt.

13 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec. Spin the plate briefly to release any bubbles trapped in the liquid.

14 Transfer the sealed plate to a thermal cycler and run the digestion program shown in Table 14, using a heated lid. After transferring the plate, click **Continue** on the prompt.

Table 14 Thermal cycler program for HaloPlex HS restriction digestion

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Preparation of each restriction digest reaction plate takes approximately 30-45 minutes.

For 12 column-runs, the two 384-well plates are prepared sequentially, for a total run time of approximately 90 minutes. Run the thermal cycler digestion program for each plate as soon as prompted. During the 30-minute incubation of plate 1 in the thermal cycler, the NGS Bravo begins preparation of the digestion reactions in plate 2. Once the thermal cycler program is complete for plate 1, store the digested DNA in plate 1 on ice until the Digestion_v1.0.pro protocol and thermal cycler program for plate 2 is finished.

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

a Transfer 4 µl of each ECD digestion reaction from the wells of the 384-well reaction plate indicated in Table 15 to fresh 0.2-ml PCR tubes. Note that for 12-column runs, four of the eight ECD digests are found on the first 384-well plate, and the remaining four digests are on the second 384-well plate.

Table 15 Position of ECD digestion reactions for obtaining validation samples

Restriction Enzyme Master Mix to be Validated	Position of ECD Digestion Reaction in 384-Well Plates	
	1-6 Column Runs	12-Column Runs (two 384-well plates produced)
RE Master Mix A	A1	A1 (plate 1)
RE Master Mix B	A2	A2 (plate 1)
RE Master Mix C	B1	B1 (plate 1)
RE Master Mix D	B2	B2 (plate 1)
RE Master Mix E	A13	A1 (plate 2)
RE Master Mix F	A14	A2 (plate 2)
RE Master Mix G	B13	B1 (plate 2)
RE Master Mix H	B14	B2 (plate 2)

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

c Analyze the prepared samples by automated electrophoresis using the Agilent 4200 TapeStation (see page 41), the Agilent 2100 Bioanalyzer (see page 42), or using gel electrophoresis (see page 43).

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 5, Figure 4 and Figure 6, 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 4200 TapeStation or 2200 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 stock solution and 1 µl of nuclease-free water.
- Prepare the TapeStation samples as instructed in the instrument user manual. Use 2 µl of each ECD sample diluted with 2 µl of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.
- 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 4 for sample TapeStation electrophoresis results.

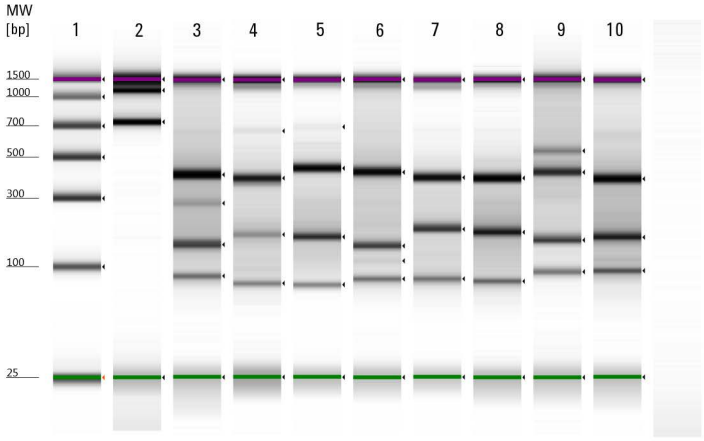


Figure 4 Validation of restriction digestion by 2200 TapeStation analysis. Lane 1: High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

Option 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 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 5 for sample Bioanalyzer electrophoresis results.

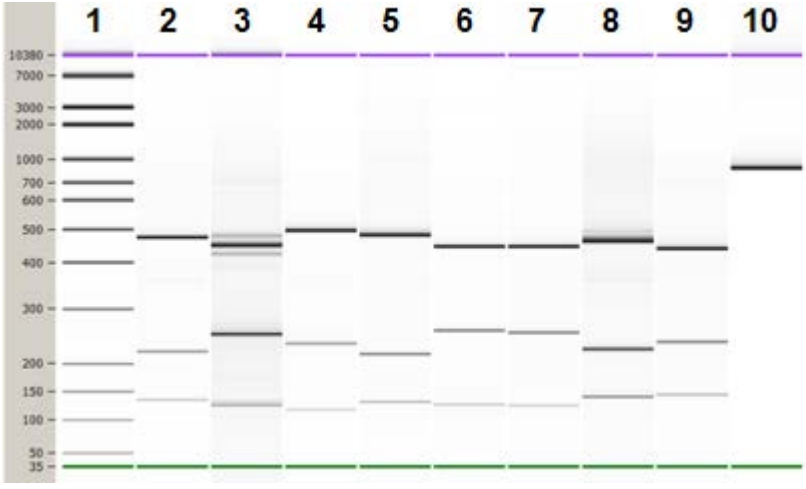


Figure 5 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

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 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 6 for sample gel results.

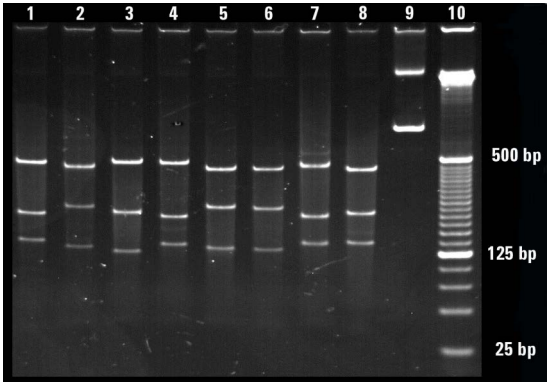


Figure 6 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

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 64.

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.

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

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a decontamination wipe.
2. Place a red insert on Bravo deck position 1.
3. Place a silver Nunc plate insert on Bravo deck position 9.
4. Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 ml of 25% ethanol.
5. For all run sizes, place a 384-well adapter insert on Bravo deck position 4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.

   For 12-column runs only, place a second 384-well adapter insert on Bravo deck position 6 and pre-set the temperature of Bravo deck position 6 to 4°C.

NOTE

To expedite thermal cycler warm-up for the hybridization reaction on page 48, you can enter and initiate the hybridization program on the thermal cycler now, and then pause the program until you are instructed to transfer the reaction plate. Be sure to pause the thermal cycler before the initiation of the 95°C incubation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 11 on page 48. Be sure that the 96-well block is in the thermal cycler before initiating the program for warm-up.

Prepare the Master Mix Source Plate for Hybridization_v1.0.pro

1 Prepare the appropriate amount of Hybridization Master Mix, according to the table below.

Mix well by gentle vortexing, then spin the tube briefly.

Table 16 Preparation of Hybridization Master Mix for Hybridization_v1.0.pro

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
HaloPlex HS Probe	5 µl	63.8 µl	106.3 µl	148.8 µl	191.3 µl	276.3 µl	552.5 µl
Hybridization Solution	34 µl	433.5 µl	722.5 µl	1011.5 µl	1300.5 µl	1878.5 µl	3757.0 µl
Total Volume	**39 µl**	**497.3 µl**	**828.8 µl**	**1160.3 µl**	**1491.8 µl**	**2154.8 µl**	**4309.5 µl**

2 In a Nunc DeepWell plate, prepare the Hybridization Master Mix source plate. Add the volumes indicated in Table 17 of the Hybridization Master Mix to all wells of Column 1 of the Nunc DeepWell plate.

Table 17 Preparation of the Master Mix Source Plate for Hybridization_v1.0.pro

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc DeepWell Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Hybridization Master Mix	Column 1 (A1-H1)	57.3 µl	98.7 µl	140.2 µl	181.6 µl	264.5 µl	533.8 µl

Prepare the Hybridization Reaction Plate with HaloPlex HS Indexing Primers

1 In a half-skirted 96-well Eppendorf twin.tec plate, aliquot 5 µl of the appropriate HaloPlex HS Indexing Primer to each intended sample indexing well position. Keep the plate on ice.

Be sure to add only one specific Indexing Primer to each well, using different indexes for each sample to be multiplexed. Record the identity of the Indexing Primer assigned to each well 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.

2 If the run includes an ECD control sample that was analyzed as described on page 39, add 32 µl of nuclease-free water to well A1 of the hybridization reaction plate. (Well A1 should also contain 5 µl of indexing primer from step 1 above.) The 32 µl of water added-back here compensates for the combined volume removed from the eight ECD digest wells during validation.

Load the Agilent NGS Bravo and Run the Hybridization_v1.0.pro VWorks Protocol

1 On the VWorks HaloPlex HS form, under **Step**, select **02 Hybridization_v1.0.pro.**

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Update layout and information.**

4 Load the Bravo deck according to Table 18.

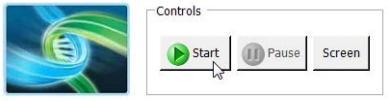
Table 18 Initial Bravo deck configuration for Hybridization_v1.0.pro

Location	Content
1	Indexing Primer source plate (half-skirted 96-well Eppendorf twin.tec plate) seated on red insert
2	New tip box
3	—(empty)—
4	Digested DNA in 384-well plate, seated on 384-well insert
5	Empty full-skirted 96-well Eppendorf twin.tec plate
6	For 12-column runs only: Digested DNA in 384-well plate (digest plate 2), seated on 384-well insert For 1- to 6-column runs: empty
7	—(empty)—
8	Empty tip box
9	Hybridization Master Mix source plate (Nunc DeepWell plate) seated on silver insert

5 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** and **Information** regions of the form.

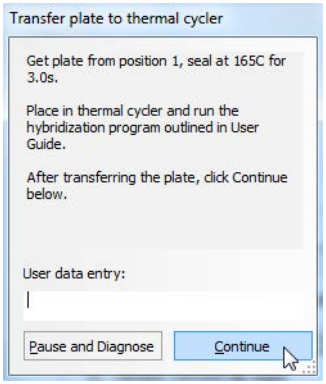
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

7 When verification is complete, click **Start** to start the run.



The NGS Bravo combines all eight digestion reactions for each gDNA sample with Hybridization Master Mix and the appropriate Indexing Primer in wells of a 96-well plate.

8 When the NGS Bravo has finished preparing the hybridization plate for the run, you will be prompted by VWorks as shown below.



9 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

10 Spin the plate briefly. The volume of each hybridization reaction is 100 µl.

11 Transfer the sealed plate to a thermal cycler and run the appropriate program in Table 19, 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.

After transferring the plate, click **Continue** on the VWorks prompt to finish the protocol.

Table 19 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

12 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 Agencourt 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. Purify then ligate the circularized DNA hybrids

In this step the hybridization buffer is removed using AMPure XP beads and then the circularized hybridization products are treated with DNA Ligase to close nicks in the probe-target DNA hybrids.

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a decontamination wipe.
2. Place a red insert on Bravo deck position 6.
3. Place the silver Nunc plate insert on Bravo deck position 9.
4. Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 ml of 25% ethanol.

Prepare the purification reagents

5. Prepare a Thermo Scientific reservoir containing 45 ml of freshly-prepared 70% ethanol.
6. Verify that the AMPure XP beads have been kept at room temperature for at least 30 minutes. Mix the room-temperature bead suspension well so that the reagent appears homogeneous and consistent in color.
7. Prepare the appropriate amount of AMPure XP beads + HS Hybridization Stop Solution mixture, according to Table 20 below. Mix the combined reagents well on a vortex mixer.

Table 20 Preparation of AMPure XP beads + HS Hybridization Stop Solution mixture

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
AMPure XP beads	0.08 ml	0.68 ml	1.32 ml	1.96 ml	2.64 ml	4.0 ml	8.0 ml
HS Hybridization Stop Solution	0.02 ml	0.17 ml	0.33 ml	0.49 ml	0.66 ml	1.0 ml	2.0 ml
Total Volume	**0.1 ml**	**0.85 ml**	**1.65ml**	**2.45 ml**	**3.3 ml**	**5 ml**	**10 ml**

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 has been dispensed from the pipette tip.

8 Prepare a Nunc DeepWell source plate containing the AMPure XP bead + HS Hybridization Stop Solution mixture from Table 20. Add 100 µl of the homogeneous bead suspension mixture to all sample wells of the Nunc DeepWell plate.

Prepare the Ligation Master Mix Source Plate

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

Prepare the amount of 1 mM rATP needed for your run size, according to Table 21.

2 Prepare the appropriate amount of Ligation Master Mix, according to Table 21.

Mix well by gentle vortexing, then spin the tube briefly.

Table 21 Preparation of Ligation Master Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	36.9 µl	470.5µl	784.1 µl	1097.8µl	1411.4 µl	2038.7 µl	4077.5 µl
1 mM rATP (from step 1)	0.6 µl	7.7 µl	12.8 µl	17.9 µl	23.0 µl	33.2 µl	66.3 µl
HS Ligation Solution	10 µl	127.5 µl	212.5 µl	297.5 µl	382.5 µl	552.5 µl	1105 µl
HS DNA Ligase	2.5 µl	31.9 µl	53.1 µl	74.4 µl	95.6 µl	138.1 µl	276.3 µl
Total Volume	**50 µl**	**637.6 µl**	**1062.5 µl**	**1487.6 µl**	**1912.5 µl**	**2762.5 µl**	**5525.1 µl**

3 Using the same Nunc DeepWell plate that was used for the Hybridization_v1.0.pro run, prepare the Master Mix source plate for Hyb_Purification_&_Ligation_v1.0.pro. Add the volume indicated in Table 22 of Ligation Master Mix to all wells of column 2 of the Nunc DeepWell plate.

Table 22 Preparation of the Master Mix Source Plate for Hyb_Purification_&_Ligation_v1.0.pro

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc DeepWell Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Ligation Master Mix	Column 2 (A2-H2)	73.4 µl	126.6 µl	179.7 µl	232.8 µl	339.1 µl	684.4 µl

Load the Agilent NGS Bravo and Run the Hyb_Purification_&_Ligation_v1.0.pro VWorks Protocol

1 On the VWorks HaloPlex HS form, under **Step**, select **03 Hyb_Purification_&_Ligation_v1.0.pro.**

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Update layout and information.**

4 Load the Bravo deck according to Table 23.

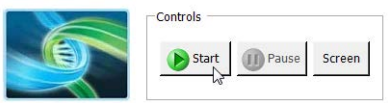
Table 23 Initial Bravo deck configuration for Hyb_Purification_&_Ligation_v1.0.pro

Location	Content
1	Empty Axygen 96 Deep Well Plate (square wells) for waste
2	New tip box
3	70% ethanol in Thermo Scientific reservoir
4	—(empty)—
5	AMPure XP beads + HS Hybridization Stop Solution mix in Nunc DeepWell source plate
6	Empty red insert
7	—(empty)—
8	Empty tip box
9	Ligation Master Mix source plate (Nunc DeepWell plate) seated on silver insert

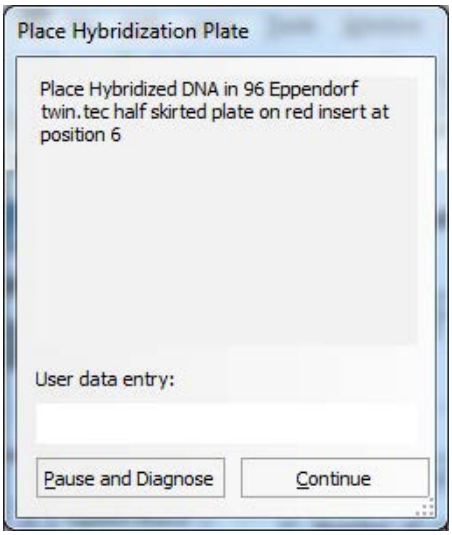
5 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** region of the form.

6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

7 When verification is complete, click **Start** to start the run.

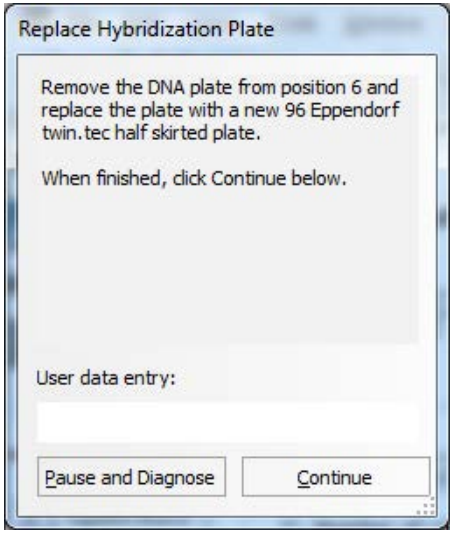


8 When prompted by VWorks as shown below, obtain the hybridization plate from the thermal cycler and spin the plate briefly to collect the liquid. Unseal the plate, then place the plate on position 6 of the Bravo deck, seated on the red insert.

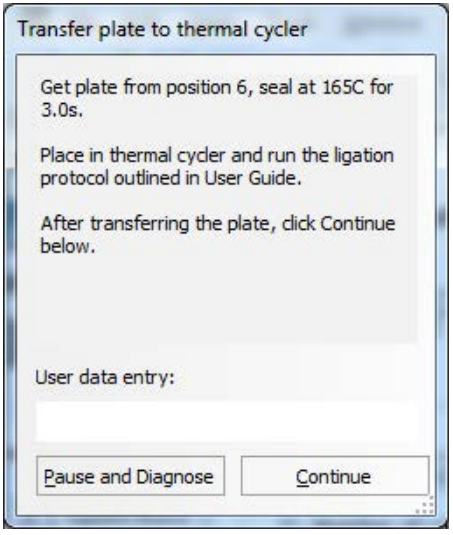


The NGS Bravo completes the liquid-handling steps for purification of the hybridized probe-target DNA.

9 When prompted by VWorks as shown below, remove and discard the hybridization plate from position 6 of the Bravo deck. Seat a fresh half-skirted 96-well Eppendorf twin.tec plate on the red insert at position 6 for use in the ligation segment of the protocol.



10 The NGS Bravo completes the liquid-handling steps for the ligation reaction. When the ligation plate is prepared, you will be prompted by VWorks as shown below.



11 Get the sample plate from position 6 and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

12 Transfer the sealed plate to a thermal cycler and incubate 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.

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

- Dynabeads MyOne Streptavidin T1 magnetic beads (described on page 56)
- Wash 1 Mix (prepare as described on page 57)

Step 4. Capture and wash the target DNA

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

Prepare the NGS Bravo

1 Gently wipe down the Bravo deck with a decontamination wipe.

2 Place a red insert on Bravo deck position 6.

Prepare the Streptavidin beads source plate

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

4 Wash the magnetic beads:

a Transfer 40 µl per sample of the Dynabeads MyOne Streptavidin T1 magnetic bead suspension to a 1.5-ml tube or conical vial, using volumes provided in Table 24.

Table 24 Volume of Dynabeads MyOne Streptavidin T1 magnetic bead suspension for capture

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Streptavidin T1 Magnetic Beads	0.04 ml	0.36 ml	0.68 ml	1.0 ml	1.32 ml	1.96 ml	3.92 ml

b Put the vial into a compatible magnetic device 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 HS Capture Solution (see Table 25) to the beads and resuspend by pipetting up and down.

Table 25 Volume of Capture Solution used for bead resuspension

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
HS Capture Solution	0.04 ml	0.36 ml	0.68 ml	1.0 ml	1.32 ml	1.96 ml	3.92 ml

5 Prepare a Nunc DeepWell source plate for the prepared streptavidin magnetic bead suspension. Add 40 µl of the homogeneous bead suspension to all sample wells of the Nunc DeepWell plate.

6 Place the streptavidin bead source plate at position 5 of the Bravo deck.

Prepare Wash 1 Mix

7 Prepare fresh 1 M NaOH for use in step 8.

Prepare the amount of 1 M NaOH solution shown in Table 26 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.

Table 26 Amount of 1 M NaOH required per run size

Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
11 µl	99 µl	187 µl	275 µl	363 µl	550 µl	1.1 ml

8 Prepare HS Wash 1 + NaOH Mix by combining the reagents in Table 27.

Table 27 Preparation of HS Wash 1 + NaOH Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
HS Wash 1 Solution	99 µl	891 µl	1683 µl	2475 µl	3267 µl	4950 µl	9.9 ml
1 M NaOH (prepared in step 7)	11 µl	99 µl	187 µl	275 µl	363 µl	550 µl	1.1 ml
Total Volume	**110 µl**	**990 µl**	**1870 µl**	**2750 µl**	**3630 µl**	**5500 µl**	**11.0 ml**

Prepare the wash solution source plates

9 Prepare a separate source plate for each of the wash solutions listed in Table 28. Use full-skirted 96-well Eppendorf twin.tec plates to prepare each source plate. For all sample-containing wells of the ligation plate, add the specified volume to all corresponding wells of each wash solution source plate.

Table 28 Preparation of solution source plates for Capture_&_Wash_v1.0.pro protocol

Solution	Volume to dispense per well of source plate
HS Wash 1 + NaOH Mix from step 8	110 µl
HS Wash 2 Solution	160 µl

10 Prepare a Thermo Scientific reservoir containing 45 ml of nuclease-free water.

Load the NGS Bravo and Run the Capture_&_Wash_v1.0.pro VWorks Protocol

1 On the VWorks HaloPlex HS form, under **Step**, select **04 Capture_&_Wash_v1.0.pro.**

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Update layout and information.**

4 Load the Bravo deck according to Table 29.

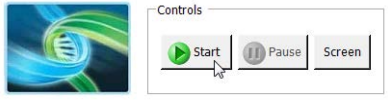
Table 29 Initial Bravo deck configuration for Capture_&_Wash_v1.0.pro

Location	Content
1	Empty Axygen 96 Deep Well Plate (square wells) for waste
2	New tip box
3	HS Wash 1 + NaOH Mix source plate (full-skirted 96-well Eppendorf twin.tec plate)
4	HS Wash 2 Solution source plate (full-skirted 96-well Eppendorf twin.tec plate)
5	Streptavidin T1 magnetic beads suspended in HS Capture Solution (Nunc DeepWell source plate)
6	Empty red insert
7	—(empty)—
8	Empty tip box
9	Nuclease-free water in Thermo Scientific reservoir

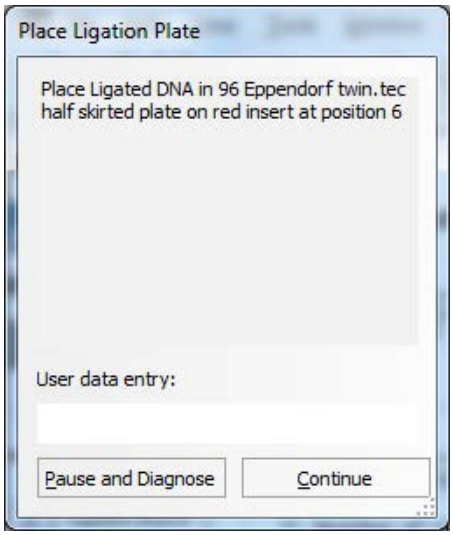
5 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** and **Information** regions of the form.

6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

7 When verification is complete, click **Start** to start the run.



8 When prompted by VWorks as shown below, obtain the ligation plate from the thermal cycler. Unseal the plate, then place the plate on position 6 of the Bravo deck, seated on the red insert.



The NGS Bravo completes the liquid-handling steps for capture of the target DNA on the streptavidin beads followed by washing of the captured DNA.

Step 5. PCR-amplify the captured target library

In this step, the Bravo completes the liquid handling steps to prepare the captured DNA target libraries for PCR amplification.

NOTE

To expedite thermal cycler warm-up for the subsequent PCR program on page 64, you can enter and initiate the PCR program on the thermal cycler now, and then pause the program until you are instructed to transfer the reaction plate. Be sure to pause the thermal cycler before the initiation of the 98°C denaturation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 15.

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a decontamination wipe.
2. Place a red insert on Bravo deck position 6.
3. Place the silver Nunc plate insert on Bravo deck position 9.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.
5. Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 ml of 25% ethanol.

Prepare the PCR Master Mix Source Plate for Amplification_v1.0.pro

6 Prepare the appropriate amount of PCR Master Mix, according to Table 30 below.

Mix well by gentle vortexing, then spin the tube briefly.

Table 30 Preparation of PCR Master Mix for Amplification_v1.0.pro

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	3.2 µl	40.8 µl	68.0 µl	95.2 µl	122.4 µl	176.8 µl	353.6 µl
Herculase II Reaction Buffer	30 µl	382.5 µl	637.5 µl	892.5 µl	1147.5 µl	1657.5 µl	3315 µl
100 mM dNTP Mix	0.8 µl	10.2 µl	17.0 µl	23.8 µl	30.6 µl	44.2 µl	88.4 µl
Primer 1	4 µl	51.0 µl	85.0 µl	119.0 µl	153.0 µl	221.0 µl	442 µl
Primer 2	8 µl	102 µl	170 µl	238 µl	306 µl	442 µl	884 µl
Herculase II Fusion DNA Polymerase	4 µl	51.0 µl	85.0 µl	119.0 µl	153.0 µl	221.0 µl	442 µl
Total Volume	**50 µl**	**637.5 µl**	**1062.5 µl**	**1487.5 µl**	**1912.5 µl**	**2762.5 µl**	**5525 µl**

7 Using the same Nunc DeepWell plate that was used for the Hyb_Purification_&_Ligation_v1.0.pro run, prepare the Master Mix source plate for Amplification_v1.0.pro. Add the volume indicated in Table 31 of PCR Master Mix to all wells of column 3 of the Nunc DeepWell plate.

Table 31 Preparation of the Master Mix Source Plate for Amplification_v1.0.pro

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
PCR Master Mix	Column 3 (A3-H3)	73.4 µl	126.6 µl	179.7 µl	232.8 µl	339.1 µl	684.4 µl

Load the Agilent NGS Bravo and Run the Amplification_v1.0.pro VWorks Protocol

8 On the VWorks HaloPlex HS form, under **Step**, select **05 Amplification_v1.0.pro.**

9 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

10 Click **Update layout and information.**

11 Load the Bravo deck according to Table 32.

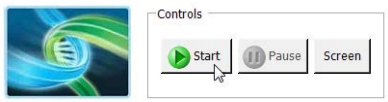
Table 32 Initial Bravo deck configuration for Amplification_v1.0.pro

Location	Content
1	—(empty)—
2	New tip box
3	—(empty)—
4	—(empty)—
5	Captured, washed DNA samples in Nunc DeepWell plate
6	Empty half-skirted 96-well Eppendorf twin.tec plate seated on red insert
7	—(empty)—
8	Empty tip box
9	Master Mix source plate (Nunc DeepWell plate) seated on silver insert

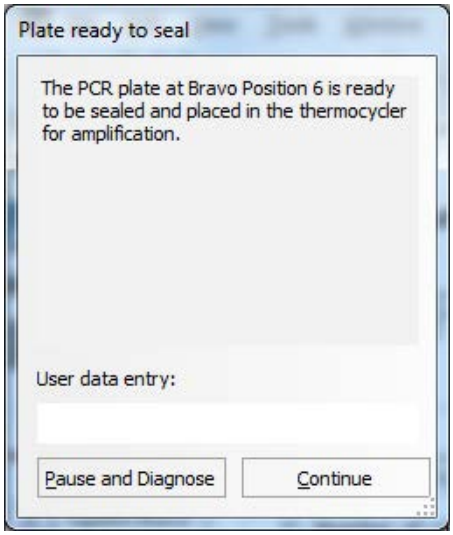
12 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** and **Information** regions of the form.

13 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

14 When verification is complete, click **Start** to start the run.



15 When the NGS Bravo has finished preparing the PCR amplification reactions, you will be prompted by VWorks as shown below.



a Get the sample plate from position 6 and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

b Transfer the sealed plate to a thermal cycler and run the PCR program in Table 33, using a heated lid.

Table 33 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

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.

c After initiating the PCR program in the thermal cycler, click **Continue** on the VWorks prompt to finish the automation protocol.

d If you are continuing to the next step of PCR product purification, remove the Agencourt AMPure XP Beads from +4°C storage for use on page 66. Let the beads come to room temperature for the remainder of the amplification program.

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 6. Purify the amplified target DNA

In this step, the NGS Bravo does the liquid handling steps to purify the amplified target DNA sample using AMPure XP beads.

Prepare the NGS Bravo and reagents

1. Gently wipe down the Bravo deck with a decontamination wipe.
2. Place a red insert on Bravo deck position 6.
3. Pre-set the temperature of Bravo deck position 6 to 4°C and position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.
4. Verify that the AMPure XP beads have been held at room temperature for at least 30 minutes.

   Do not freeze the AMPure XP beads at any time.
5. Mix the room-temperature bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate containing AMPure XP beads mixed with nuclease-free water.
   a. For each well to be processed, add 100 µl of homogeneous AMPure XP beads per well.
   b. Add 40 µl of nuclease-free water to each well of beads in the Nunc DeepWell plate.
7. Prepare a Thermo Scientific reservoir containing 45 ml of freshly-prepared 70% ethanol.
8. Prepare the elution buffer source plate by placing 45 µl of HS Elution Buffer in each sample well of a full-skirted 96-well Eppendorf twin.tec plate.

Load the Agilent NGS Bravo and Run the Final_Purification_v1.0.pro VWorks Protocol

1. On the VWorks HaloPlex HS form, under **Step**, select **06 Final_Purification_v1.0.pro.**
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Click **Update layout and information.**

4 Load the Bravo deck according to Table 34.

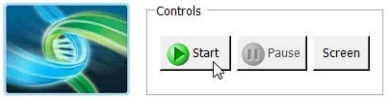
Table 34 Initial Bravo deck configuration for Final_Purification_v1.0.pro

Location	Content
1	Empty Axygen 96 Deep Well Plate (square wells) for waste
2	New tip box
3	HS Elution Buffer in full-skirted 96-well Eppendorf twin.tec plate
4	—(empty)—
5	AMPure XP beads + water mixture in Nunc DeepWell source plate
6	Amplified DNA samples in half-skirted 96-well Eppendorf twin.tec plate seated on red insert
7	—(empty)—
8	Empty tip box
9	70% ethanol in Thermo Scientific reservoir

5 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** region of the form.

6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

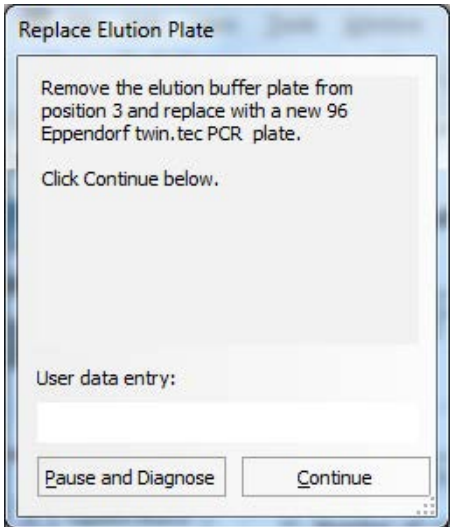
7 When verification is complete, click **Start** to start the run.



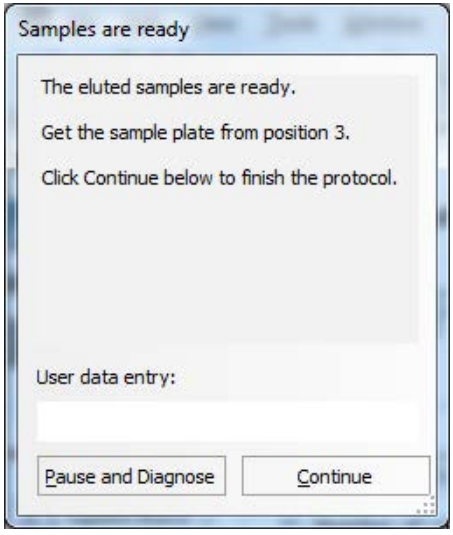
The NGS Bravo completes the liquid-handling steps for purification of the amplified target DNA.

8 When prompted by VWorks as shown below, remove and discard the elution buffer source plate from position 3 of the Bravo deck. Place a fresh full-skirted 96-well Eppendorf twin.tec plate on position 3.

When finished, click **Continue** on the VWorks prompt.



9 When the NGS Bravo has finished preparing the final eluted sample plate, you will be prompted by VWorks as shown below. Click **Continue** on the VWorks prompt to finish the protocol.



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 7. 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 71) or 2100 Bioanalyzer (see page 72).

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 7.

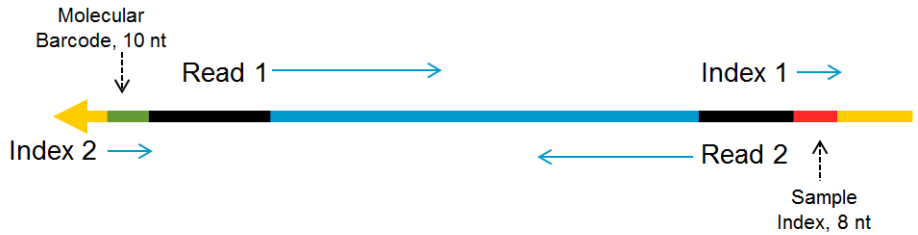


Figure 7 Content of HaloPlex HS-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red), the molecular barcode (green) and the library bridge PCR primers (yellow).

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 Assay 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 TapeStation as instructed in the user manual. Start the run.

3 Analyze the electropherogram for each sample using the analysis guidelines on page 73.

See Figure 8 for a sample TapeStation electropherogram.

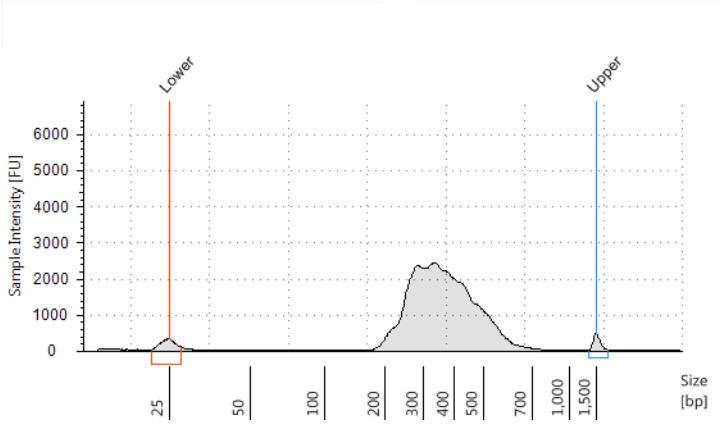


Figure 8 Validation of HaloPlex HS enrichment by 2200 TapeStation analysis.

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 73.

See Figure 9 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.

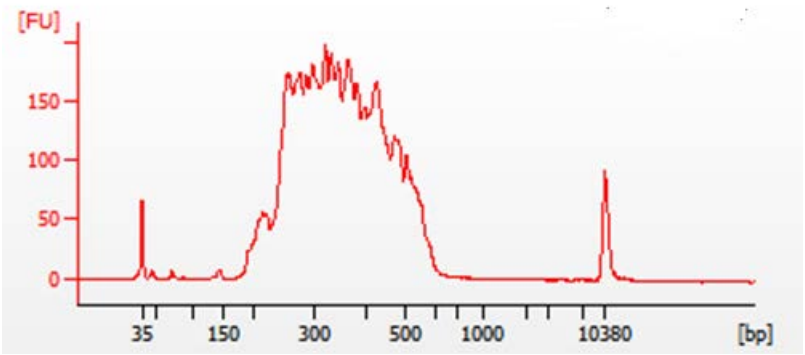


Figure 9 Validation of HaloPlex HS enrichment by 2100 Bioanalyzer analysis.

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 84.

Step 8. 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 75 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.

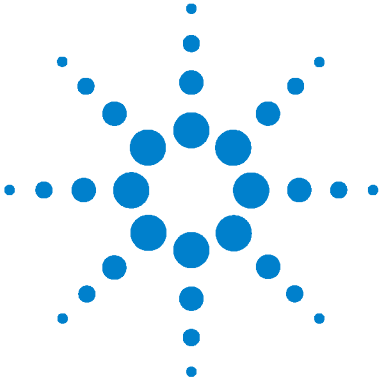
MiSeq platform sequencer setup requirements

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.



4 Reference

Kit Contents

Component kits supplied with the HaloPlex HS Target Enrichment System are listed in Table 35. Reagents included in the kits are listed in Table 36 on page 78.

Table 35 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 36 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.

Table 36 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 33 to page 34)

† See Table 37 for a plate map.

‡ See Table 38 for a plate map.

Table 37 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 38 Plate map for HaloPlex HS Indexing Primers A01 through H12 provided with 96-reaction kits

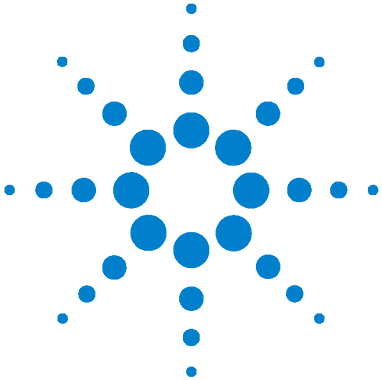
	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	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 39 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



5 Appendix I: 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 40.

Table 40 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 41 to determine the recommended amount of sequencing output for each HaloPlex HS-enriched FFPE sample based on the ΔΔCq DNA integrity score.

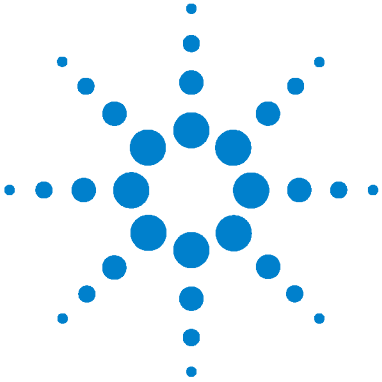
Table 41 Recommended sequencing augmentation for FFPE-derived DNA samples

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.



6 Appendix II: Provisional Adaptor-Dimer Removal Protocol

This section contains a protocol for purification of the target-enriched library pool to remove adaptor-dimer molecules of approximately 140 bp size. Only do this protocol if electrophoretic analysis of the target-enriched library samples shows a peak at approximately 140 bp which represents a molar fraction of >10% of DNA in the sample (see page 72 to page 73.)

Purify the enriched library pool using AMPure XP beads

In this step, a 40-µL pool of target-enriched DNA libraries is purified using AMPure XP beads using manual sample processing.

NOTE This protocol requires a 0.2 ml tube-compatible magnetic separation device, such as the Agencourt SPRIPlate Super Magnet Plate, Agencourt p/n A32782, or equivalent.

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 target-enriched library pool to a fresh 0.2-ml tube.
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 homogeneous AMPure XP bead suspension. Mix well, until the bead mix suspension appears homogeneous.
6. Add 140 µL of the homogeneous bead suspension prepared in step 5 to each 40-µL DNA 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 separation device. Wait for the solution to clear (approximately 5 minutes).
9. Keep the tubes in the magnetic device. 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 device 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 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.

15 Remove tubes from the magnetic device and add 40 µL of HS Elution Buffer to each sample.

NOTE Use room-temperature HS Elution Buffer 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 device 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.

In This Book

This guide contains information to run the HaloPlex HS Target Enrichment System automation protocol.

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