HaloPlex Target Enrichment System

Automation Protocol

For Illumina Sequencing

Protocol

Version H0, 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

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CAUTION

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In this Guide...

This guide describes an optimized automation protocol for using the HaloPlex 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 Target Enrichment

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

3 Sample Preparation

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

4 Appendix: Provisional Adaptor-Dimer Removal Protocol

This chapter describes a protocol used to remove adaptor-dimer (125 bp) molecules that may be observed for some designs.

5 Reference

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

What's New in Version H0

- 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
- Removal of recommendation for hybridization using the SureCycler thermal cycler (see Table 3 on page 11 and page 48)
- Updates to lists of available HaloPlex kits in Table 2 on page 10 and Table 33 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 69 to page 70
- Removal of support information for ClearSeq pre-designed probes throughout this publication
- Updates to information on use of Agilent's SureDesign for access to HaloPlex probe designs and kit ordering information on page 10 and page 30
- Updates to Agilent.com webpage hyperlinks
- Updates to downstream sequencing support information on page 72

Content

5 Reference

1 Before You Begin

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

Procedural Notes

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

Safety Notes

CAUTION

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

Required Reagents

Table 1 Required Reagents for HaloPlex Target Enrichment

Description	Vendor and part number
HaloPlex Target Enrichment System Kit	Select the appropriate kit for your probe design from Table 2
Herculase II Fusion Enzyme with dNTPs (100 mM; 25 mM for each nucleotide), 200 reactions	Agilent p/n 600677
Nuclease-free Water (not DEPC-treated)	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
10 M NaOH, molecular biology grade	Sigma, p/n 72068
2 M acetic acid	Fluka, p/n A8976
10 mM Tris-HCl, pH 8.0 or 10 mM Tris-acetate, pH 8.0	General laboratory supplier
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer 100 assays, 2-1000 ng 500 assays, 2-1000 ng	 Thermo Fisher Scientific p/n Q32850 Thermo Fisher Scientific p/n Q32853

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

Table 2 HaloPlex Target Enrichment System Kits for Illumina Sequencing

Probe Design	Part Number-96 Reactions
HaloPlex Custom Panel Tier 1*, ILMFST	G9901B
HaloPlex Custom Panel Tier 1 Plus*, ILM	G9961B
HaloPlex Custom Panel Tier 2†, ILM	G9911B
HaloPlex Custom Panel Tier 3‡, ILM	G9921B

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

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

‡ Tier 3 designs are 2.6 Mb-5 Mb.

NOTE

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

Required Equipment

Table 3 Required Equipment for HaloPlex 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 Fisher Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL volume	Thermo Fisher 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 Fluorometer	Thermo Fisher Scientific p/n Q33226
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 54 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 Automation

HaloPlex target enrichment protocols are 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 verify 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 Target Enrichment

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

About the 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 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.

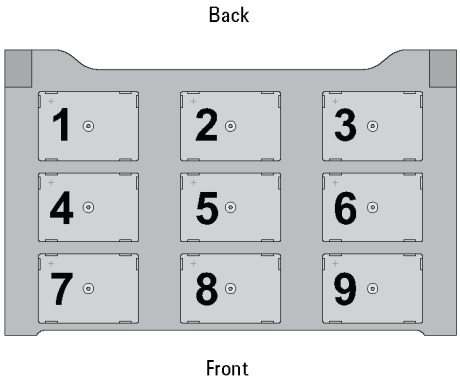


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

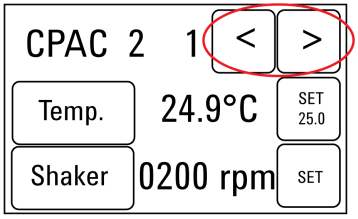
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include low-temperature (4°C) or high-temperature (54°C) incubation steps may be expedited by pre-setting the temperature of the affected block before starting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

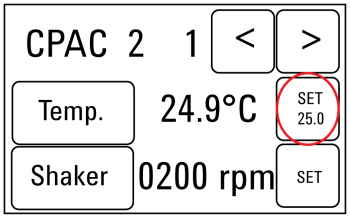
Table 7 Inheco Multi TEC Control touchscreen designations

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

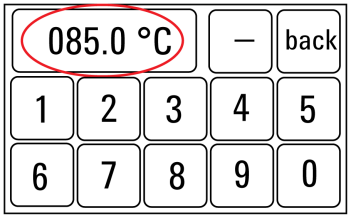
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



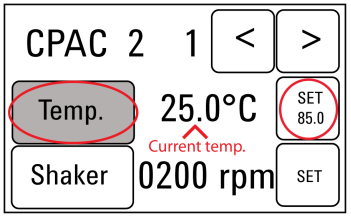
2 To set the temperature of the selected block, press the SET button.



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 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 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.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.)

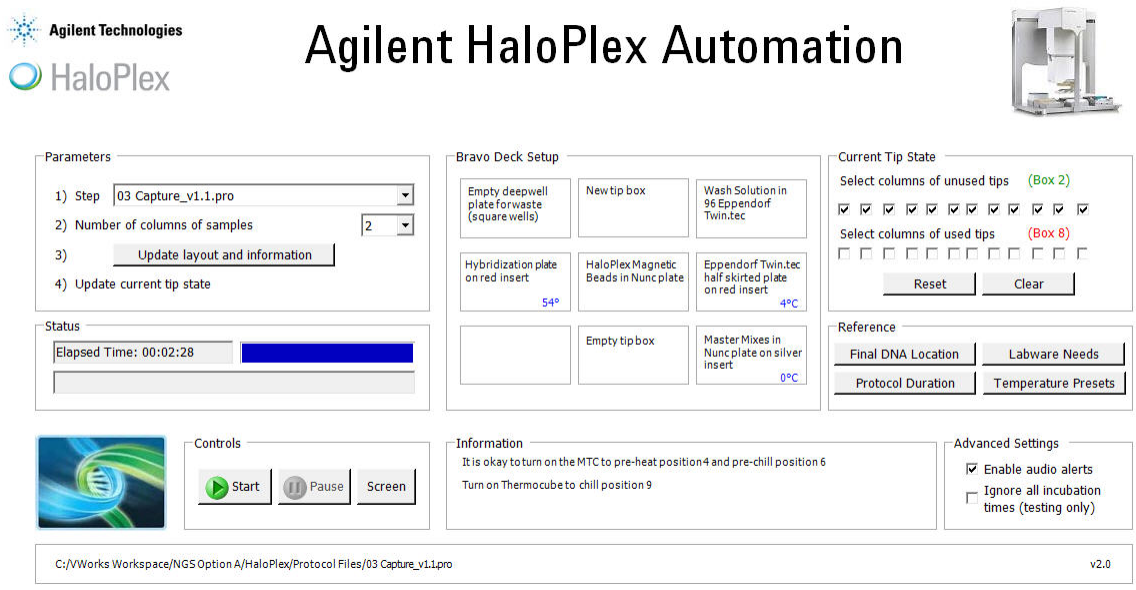
Using the HaloPlex.VWForm to setup and start a run

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

1. Open the form using the HaloPlex.VWForm shortcut on your desktop.
2. Use the drop-down menus on the form to select the appropriate HaloPlex 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.**

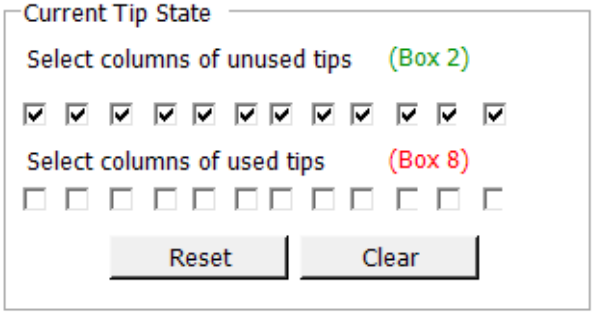
NOTE The displayed protocol will not run unless the **Update layout and information** button has been clicked.

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.

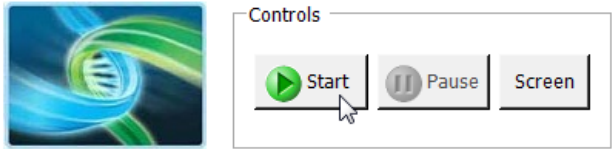
For an empty tip box, all positions of the **Current Tip State** used tip indicator (bottom portion, Box 8) should be cleared, as shown above. Clicking **Reset** clears all columns for position 8.

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 automation protocols, as long as positions of available tips are accurately indicated during run setup.

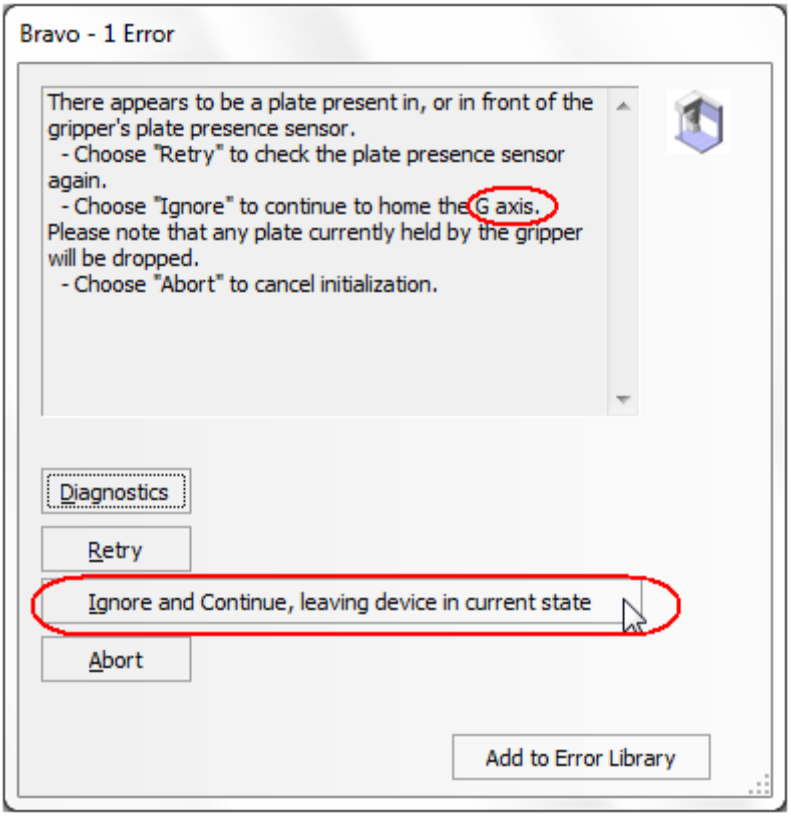
6 After verifying that the NGS Bravo has been set up correctly, click **Start** in the **Controls** section of the form to begin the run. Do not use the Start button on the VWorks Control Toolbar; runs must be initiated using the start button on the HaloPlex.VW Form, shown below.



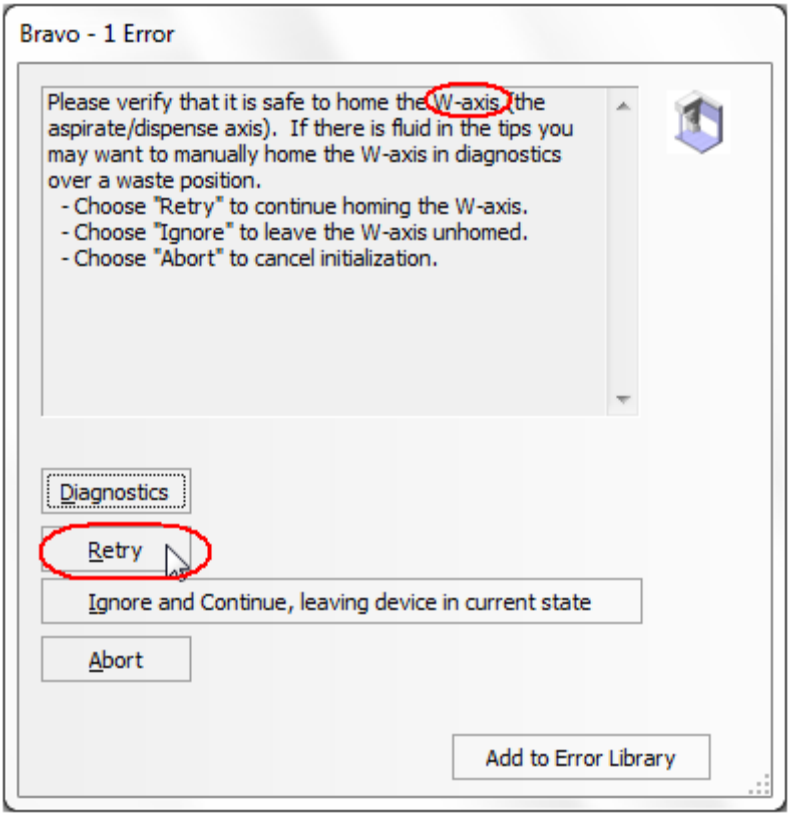
Error messages encountered at start of run

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**.



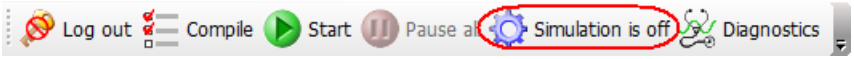
2 If you encounter the W-axis error message shown below, select **Retry.**



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. 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 Target Enrichment Procedure

Figure 2 summarizes the HaloPlex target enrichment workflow. For each sample to be sequenced, individual HaloPlex-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 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.pro
Hybridize to HaloPlex or ClearSeq probe and index samples	Hybridization.pro
Capture and amplify enriched DNA	Capture_v1.1.pro
Purify amplified libraries	Purification_v1.1.pro

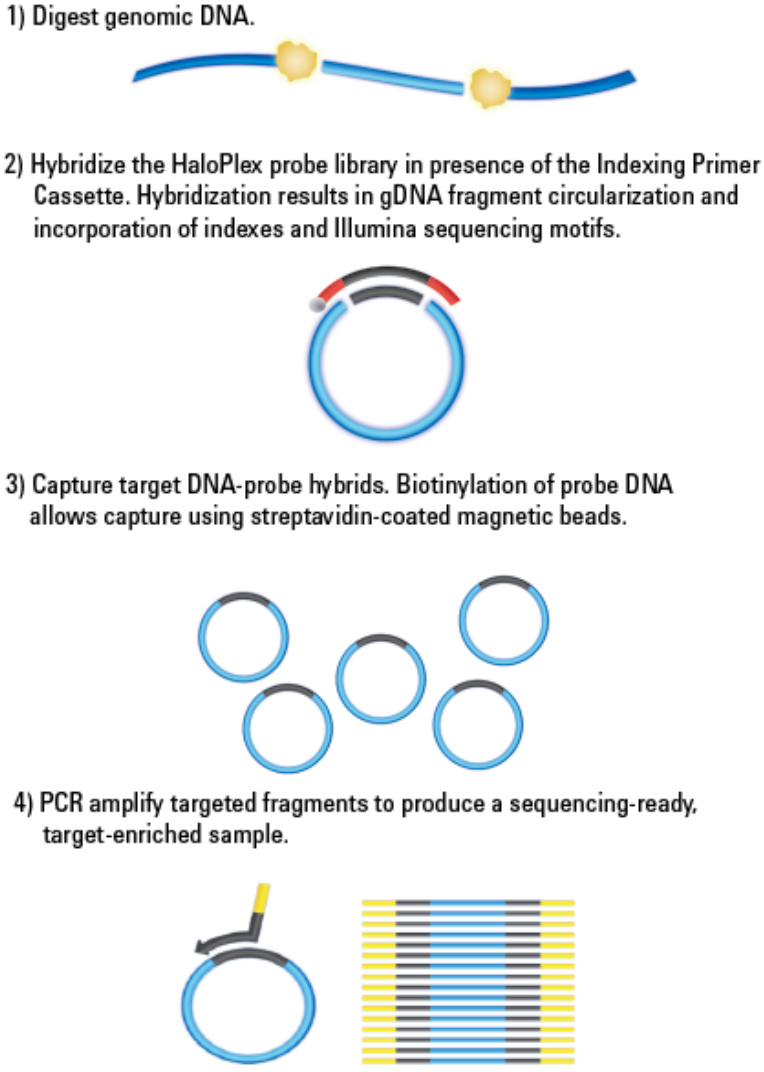


Figure 2 Overall HaloPlex target-enriched sequencing sample preparation workflow.

Experimental Setup Considerations for Automated Runs

HaloPlex 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 or ClearSeq probe (see Figure 2), you will need to prepare a separate plate containing the HaloPlex 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 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 Box 1 of your kit to determine the hybridization duration appropriate for your design. After reviewing the duration of this and other steps in the protocol, plan the start time for your experiment accordingly.

Designs containing <15,000 probes use a 3-hour hybridization time. 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 ≥15,000 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 form in the VWorks software (HaloPlex.VWForm), to allow overnight hybridization.

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. HaloPlex probe design information is available at Agilent's SureDesign website using the *Find Designs* tab.

The HaloPlex Target Enrichment System amplifies thousands of targets in the same reaction, incorporating standard Illumina paired-end sequencing motifs in the process. During hybridization, each sample can be uniquely indexed, allowing for pooling of up to 96 samples per sequencing lane.

Step 1. Digest genomic DNA with restriction enzymes

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

NOTE

Successful enrichment using the protocol in this guide requires high-quality DNA samples. Before you begin, verify that the genomic DNA samples have an OD 260/280 ratio ranging from 1.8 to 2.0. Verify the size distribution of DNA in each DNA preparation by gel electrophoresis. Any smearing below 2.5 kb indicates sample degradation.

For HaloPlex target enrichment of FFPE-derived DNA samples, see Agilent publication G9900-90050. This publication provides a PCR-based protocol for assessment of DNA integrity and provides HaloPlex protocol modifications for improved performance from lower-quality DNA samples.

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a surface 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

NOTE

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

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

Follow the manufacturers instructions for the kits and instruments.

2 Prepare the DNA 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 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 45 µL of the supplied Enrichment Control DNA (ECD). Store on ice.

b In separate wells of the same 96-well twin.tec plate, dilute 225 ng of each gDNA sample in 45 µL nuclease-free water, for a final DNA concentration of 5 ng/µL. Continue to store on ice.

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

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.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	34 µL	408 µL	680 µL	952 µL	1224 µL	1768 µL	3536 µL
re-Albumin Solution*	0.85 µL	10.2 µL	17 µL	23.8 µL	30.6 µL	44.2 µL	88.4 µL
Total Volume	**34.85 µL**	**418.2 µL**	**697 µL**	**975.8 µL**	**1254.6 µL**	**1812.2 µL**	**3624.4 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 Box 1. 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.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	4.0	51.0 µL	85.0 µL	119.0 µL	153.0 µL	221.0 µL	442.0 µL
Green Enzyme Strip enzyme A–H	0.5	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.25 µL
Red Enzyme Strip enzyme A–H	0.5	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.25 µL
Total Volume for each Master Mix A, B, C, D, E, F, G, or H	**5 µL**	**63.8 µL**	**106.2 µL**	**148.8 µL**	**191.2 µL**	**276.2 µL**	**552.5 µL**

NOTE For 1-4 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 6- or 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.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)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix B	Column 2 (A2-H2)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix C	Column 3 (A3-H3)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix D	Column 4 (A4-H4)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix E	Column 5 (A5-H5)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix F	Column 6 (A6-H6)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix G	Column 7 (A7-H7)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL
RE Master Mix H	Column 8 (A8-H8)	7.3 µL	12.7 µL	18.0 µL	23.3 µL	33.9 µL	68.4 µL

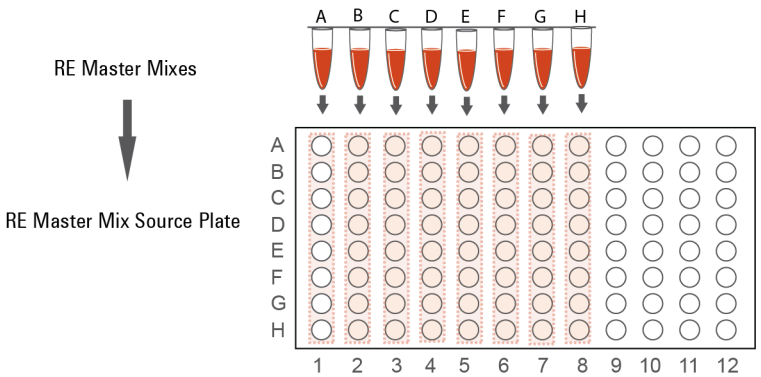


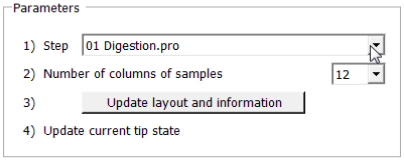
Figure 3 Preparation of the RE Master Mix source plate for automation protocol Digestion.pro.

Load the NGS Bravo and Run the Digestion.pro VWorks Protocol

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

2 Log in to the VWorks software.

3 On the setup form, under **Step**, select **01 Digestion.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.

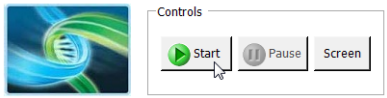
Table 13 Initial Bravo deck configuration for Digestion.pro

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.

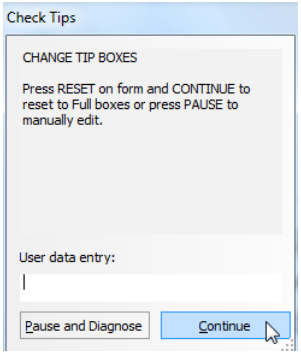
9 When verification is complete, click **Start** to start the run.



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.

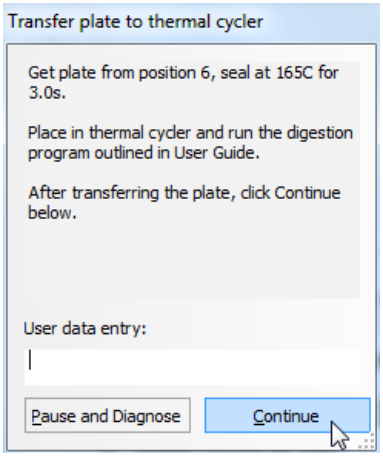
Depending on the run size, you may be prompted to change tip boxes multiple times during the run.



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 restriction digestion

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	8°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.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 by 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 4, Figure 5 and Figure 6, does not impact enrichment results.

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

Option 1: Validation by Agilent 4200 TapeStation analysis

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

- Prepare an undigested DNA gel control by combining 1 µL of the Enrichment Control DNA stock solution and 1 µL of nuclease-free water.
- Prepare the TapeStation samples as instructed in assay Quick Guide. Use 2 µL of each ECD sample diluted with 2 µL of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.
- Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 4200 TapeStation and start the run.

See Figure 4 for sample TapeStation electrophoresis results.

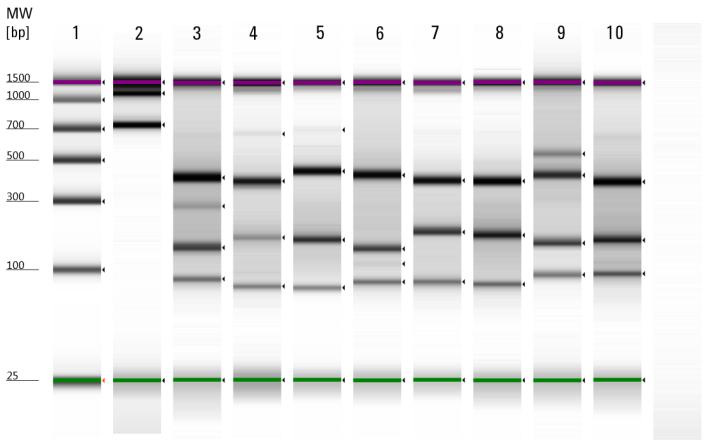


Figure 4 Validation of restriction digestion by TapeStation analysis. Lane 1: High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

Option 2: Validation by Agilent 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.

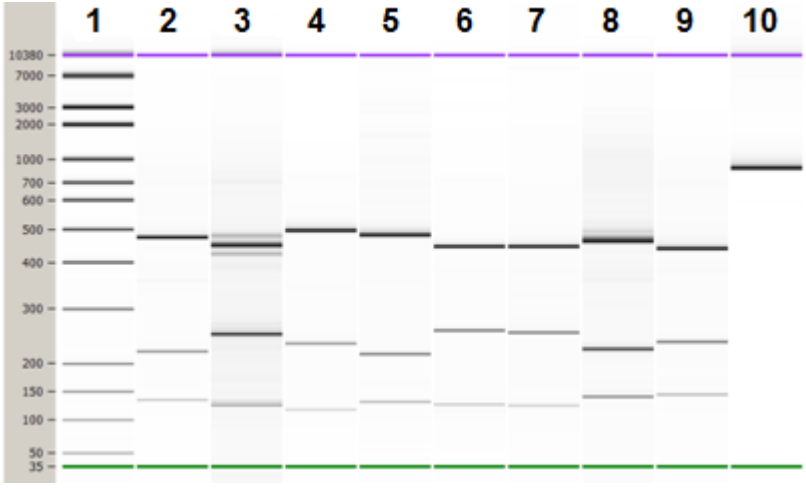


Figure 5 Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

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.

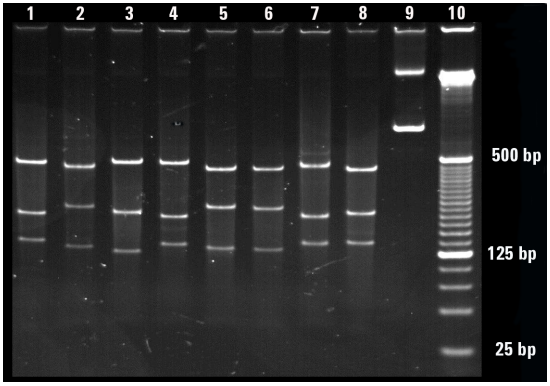


Figure 6 Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

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 63.

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

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex probe. HaloPlex probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences are incorporated into the targeted fragments.

The duration of the hybridization reaction is determined by the probe density of your design. Refer to the Certificate of Analysis provided with Box 1 of your kit to determine the hybridization conditions appropriate for your design.

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

Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a surface 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.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.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 Probe	20 µL	255 µL	425 µL	595 µL	765 µL	1105 µL	2210 µL
Hybridization Solution	50 µL	637.5 µL	1062.5 µL	1487.5 µL	1912.5 µL	2762.5 µL	5525 µL
Total Volume	**70 µL**	**892.5 µL**	**1487.5 µL**	**2082.5 µL**	**2677.5 µL**	**3867.5 µL**	**7735 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 the indicated column of the Nunc DeepWell plate.

Table 17 Preparation of the Master Mix Source Plate for Hybridization.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)	102.8 µL	177.2 µL	251.6 µL	325.9 µL	474.7 µL	958.1 µL

Prepare the Hybridization Reaction Plate with Indexing Primers

1 In a half-skirted 96-well Eppendorf twin.tec plate, aliquot 10 µL of the appropriate HaloPlex 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 index assigned to each well for later sequence analysis.

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 10 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.pro VWorks Protocol

1 On the VWorks HaloPlex form, under **Step**, select **02 Hybridization.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.

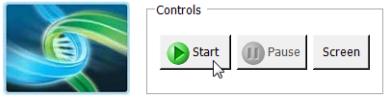
Table 18 Initial Bravo deck configuration for Hybridization.pro

Location	Content
1	HaloPlex 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.

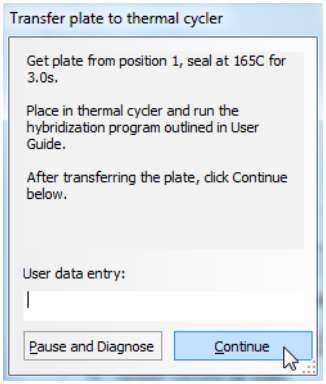
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

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.

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

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

Table 19 Thermal cycler program* for probe hybridization

Step	Temperature	Time (Duration of Step)	
		Designs with <15,000 probes (see Certificate of Analysis)	Designs with ≥15,000 probes (see Certificate of Analysis)
Step 1	95°C	10 minutes	10 minutes
Step 2	54°C	3 hours	16 hours

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

CAUTION Make sure that the thermal cycler has a maximum reaction volume specification of at least 100 µL. The performance of the selected thermal cycler in this application should be verified before use.

Step 3. Capture and amplify the target DNA

In this step, the circularized target DNA-probe hybrids, containing biotin, are captured on streptavidin beads. After capture, DNA ligase is added to seal nicks, then target DNA is eluted and PCR-amplified.

Assemble reagents for the run

1 Remove reagents to be used in upcoming protocol steps from cold storage and allow the solutions to reach room temperature:

- From –20°C storage, remove the Capture Solution, Wash Solution, Ligation Solution and SSC Buffer.
- From +4°C storage, remove the HaloPlex Magnetic Beads.

2 Prepare 30 µL per sample, plus excess, of fresh 50 mM NaOH for use in the DNA elution step on page 51.

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

CAUTION

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

- Do not use stock NaOH solutions that were stored at concentrations below 10 M to prepare the 50 mM NaOH solution.
- Keep the 50 mM NaOH solution container sealed when not in use, especially when processing large numbers of samples per run.

Table 20 Amount of 50mM 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
30 µL	270 µL	510 µL	750 µL	990 µL	1470 µL	2940 µL

3 Obtain or prepare 0.5 µL per sample, plus excess, of 2 M acetic acid, for use in the PCR master mix on page 52.

CAUTION

It is critical to use high-quality acetic acid at 2 M concentration in this step to ensure neutralization of the NaOH used for elution.

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

Prepare the NGS Bravo

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

2 Place a red insert on Bravo deck position 4.

3 Pre-set the temperature of Bravo deck position 4 to 54°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.

4 Place a second red insert on Bravo deck position 6.

5 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.

6 Place the silver Nunc plate insert on Bravo deck position 9.

7 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 HaloPlex Magnetic Beads Source Plate

1 Vigorously resuspend the HaloPlex Magnetic Beads on a vortex mixer. The beads settle during storage.

2 Wash the magnetic beads.

a Transfer 40 µL per sample of the HaloPlex Magnetic Beads suspension to a 1.5-mL tube or conical vial, using volumes provided in Table 21.

Table 21 Volume of HaloPlex 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
HaloPlex 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 Capture Solution (see Table 22) to the beads and resuspend by pipetting up and down.

Table 22 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
Capture Solution	0.04 mL	0.36 mL	0.68 mL	1.0 mL	1.32 mL	1.96 mL	3.92 mL

3 Prepare a Nunc DeepWell source plate for the washed HaloPlex streptavidin bead suspension. Add 40 µL of the homogeneous bead suspension to all wells of the Nunc DeepWell plate that correspond to sample-containing wells on the hybridization plate.

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

Prepare wash and elution solution source plates

Prepare a separate source plate for each of the solutions listed in Table 23. Use full-skirted 96-well Eppendorf twin.tec plates to prepare all three source plates. For all sample-containing wells of the hybridization plate, add the specified volume of solution to all corresponding wells of the solution source plate.

Table 23 Preparation of solution source plates for Capture_v1.1.pro protocol

Solution	Volume to dispense per well of source plate
Wash Solution	110 µL
SSC Buffer	110 µL
50 mM NaOH	30 µL

Seal the 50 mM NaOH source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Leave sealed until you are prompted to add the plate to the Bravo deck in step 15 on page 61.

Prepare the Master Mixes for Capture_v1.1.pro protocol

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

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

Table 24 Preparation of PCR Master Mix for Capture_v1.1.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	16.1 µL	205.3 µL	342.1 µL	479 µL	615.8 µL	889.5 µL	1779 µL
5X Herculase II Reaction Buffer	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105 µL
dNTPs (100 mM)*	0.4 µL	5.1 µL	8.5 µL	11.9 µL	15.3 µL	22.1 µL	44.2 µL
Primer 1	1 µL	12.75 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Primer 2	1 µL	12.75 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
2 M Acetic acid	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.3 µL
Herculase II Fusion DNA Polymerase	1 µL	12.75 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**30 µL**	**382.5 µL**	**637.6 µL**	**892.7 µL**	**1147.6 µL**	**1657.6 µL**	**3315 µL**

* Be sure to use dNTPs at 100 mM concentration (25 mM for each nucleotide), like those provided with the Herculase II Fusion Enzyme with dNTPs (Agilent p/n 600677 or 600679).

2 Prepare the appropriate amount of Ligation Master Mix, according to the table below.

Mix well by gentle vortexing, then spin the tube briefly. Store the master mix on ice until it is used on page 57. The Ligation Master Mix is added to the Master Mix Source Plate just before it is used in the Capture_v1.1.pro protocol. Do not add this master mix to the source plate before starting the run.

Table 25 Preparation of Ligation Master Mix for Capture_v1.1.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
Ligation Solution	47.5 µL	605.6 µL	1009 µL	1413 µL	1817 µL	2624 µL	5249 µL
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**	**637.5 µL**	**1062.1 µL**	**1487.4 µL**	**1912.6 µL**	**2762.1 µL**	**5525.3 µL**

Prepare the Master Mix Source Plate for Capture_v1.1.pro

Using the same Nunc DeepWell plate that was used for the Hybridization.pro run, prepare the Master Mix source plate for Capture_v1.1.pro. Add the volume indicated in Table 26 of PCR Master Mix to all wells of column 3 of the Nunc DeepWell plate.

Table 26 Preparation of the Master Mix Source Plate for Capture_v1.1.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)	44.1 µL	75.9 µL	107.8 µL	139.7 µL	203.4 µL	410.6 µL

NOTE

Column 2 of the Master Mix source plate must remain empty at this step. You will be prompted to add Ligation Master Mix to Column 2 at the appropriate time during the Capture_v1.1.pro protocol. Column 1 was used during the Hybridization.pro protocol.

If you are using a new DeepWell plate for the Capture_v1.1.pro Master Mix source plate, be sure to leave columns 1 and 2 empty at this time, adding the PCR Master Mix to column 3 of the new plate.

Load the Agilent NGS Bravo and Run the Capture_v1.1.pro VWorks Protocol

1 On the VWorks HaloPlex form, under **Step**, select **03 Capture_v1.1.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 27.

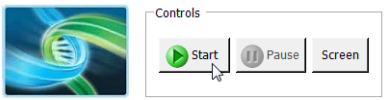
Table 27 Initial Bravo deck configuration for Capture_v1.1.pro

Location	Content
1	Empty Axygen 96 Deep Well Plate (square wells) for waste
2	New tip box
3	Wash Solution source plate (full-skirted 96-well Eppendorf twin.tec plate)
4	Hybridized sample plate seated on red insert
5	HaloPlex magnetic streptavidin bead source plate (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

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.

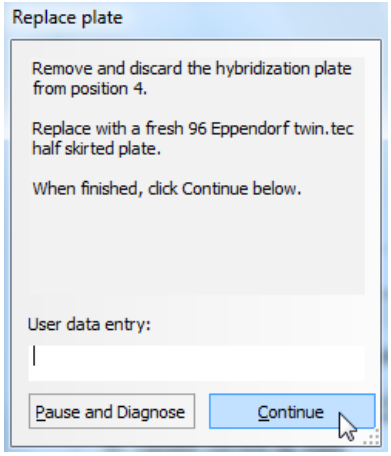


The NGS Bravo completes the liquid-handling steps for capture of the target DNA-HaloPlex probe hybrids on the streptavidin beads.

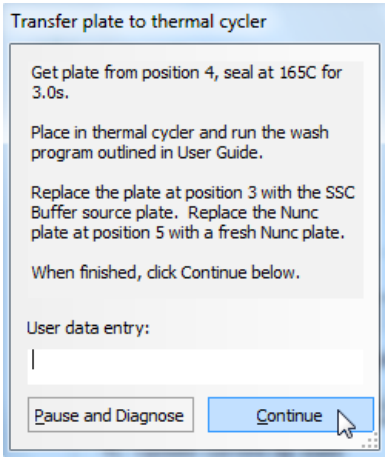
NOTE

To expedite thermal cycler warm-up for the subsequent wash program on page 56, you can enter and initiate the wash 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 46°C incubation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 9.

8 When prompted by VWorks as shown below, remove and discard the hybridization plate from position 4 of the Bravo deck. Place a fresh half-skirted 96-well Eppendorf twin.tec plate at position 4 for use in the wash segment of the protocol.



9 When the NGS Bravo has finished preparing the capture wash plate, you will be prompted by VWorks as shown below.



a Get the sample plate from position 4 and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

Transfer the sealed plate to a thermal cycler and run the wash program shown in Table 28, using a heated lid.

Do **not** include a low-temperature hold step in the thermal cycler program following the 10-minute incubation.

Table 28 Thermal cycler program for Capture_v1.1.pro wash step

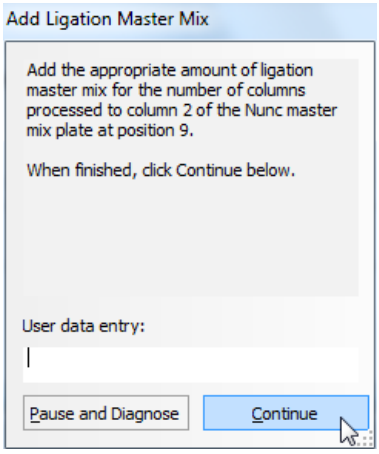
Step	Temperature	Time
Step 1	46°C	10 minutes

b Remove and discard the Wash Solution plate from position 3 of the Bravo deck. Place the SSC Buffer source plate at position 3 for use in the ligation segment of the protocol.

c Remove and discard the bead source plate from position 5 of the Bravo deck. Place a fresh Nunc DeepWell plate at position 5.

d After completing all steps, click **Continue** on the VWorks prompt to continue the automation protocol. Do not wait for the conclusion of the thermal cycler wash program to continue the protocol.

10 During the 10-minute incubation of the sample plate on the thermal cycler, you will be prompted to add the Ligation Master Mix to the Master Mix source plate as shown below.



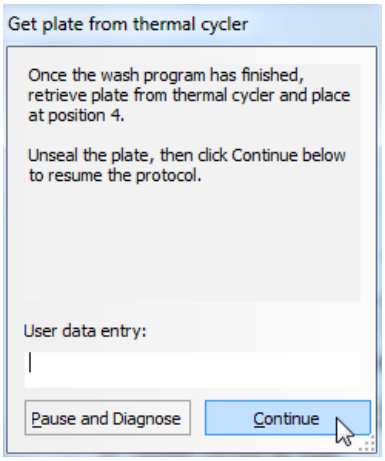
Add the volume of Ligation Master Mix indicated in Table 29 to all wells of column 2 of the Nunc DeepWell Master Mix source plate on Bravo deck position 9.

Table 29 Addition of Ligation Master Mix to the Master Mix Source Plate for Capture_v1.1.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
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

NOTE The Master Mix source plate at postition 9 should already contain the PCR Master Mix in Column 3 and the depleted Hybridization Master Mix from the Hybridization.pro protocol in Column 1. Be sure to add the Ligation Master Mix to Column 2 of the source plate at this step.

11 Once the wash program in Table 28 has finished and you are prompted by VWorks, transfer the plate from the thermal cycler to Bravo deck position 4. Carefully unseal the plate, then click Continue on the VWorks prompt to resume the Capture_v1.1.pro protocol.

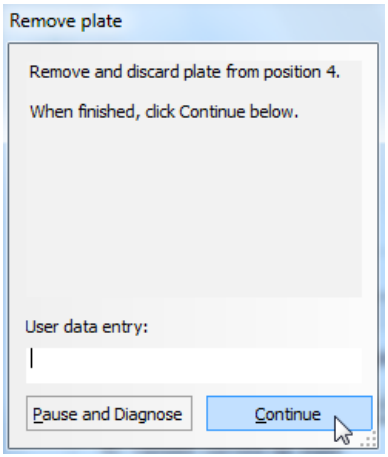


The NGS Bravo completes the liquid-handling steps for ligation of the captured target DNA.

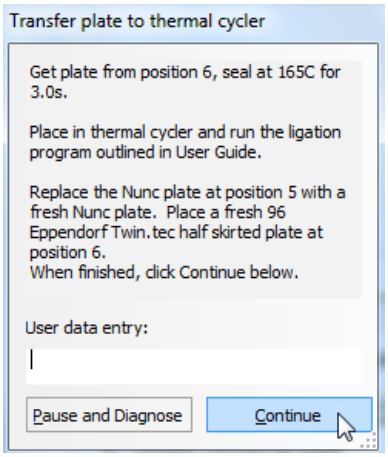
NOTE

To expedite thermal cycler warm-up for the subsequent ligation program on page 60, you can enter and initiate the wash 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 55°C incubation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 13.

12 When prompted by VWorks as shown below, remove and discard the plate from position 4. Click Continue on the prompt to resume the protocol.



13 When the NGS Bravo has finished preparing the ligation plate, 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 ligation program shown in Table 30, using a heated lid.

Table 30 Thermal cycler program for Capture_v1.1.pro ligation step

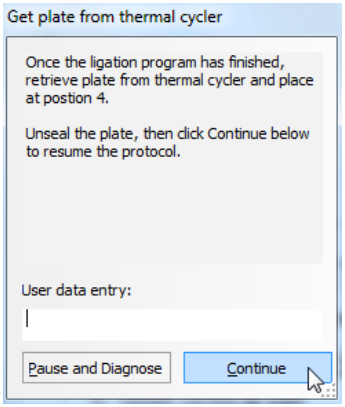
Step	Temperature	Time
Step 1	55°C	10 minutes
Step 2	4°C	Hold

c Remove and discard the Nunc DeepWell plate from position 5 of the Bravo deck. Place a fresh Nunc DeepWell plate at position 5 for use in the ligation purification steps of the protocol.

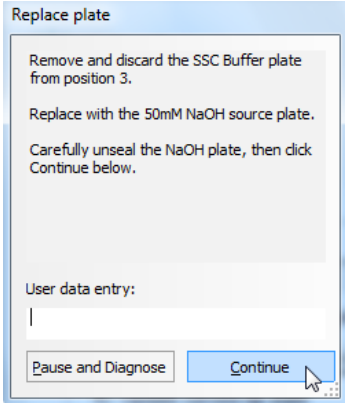
d Place a fresh half-skirted 96-well Eppendorf twin.tec plate at position 6 for use in the following PCR protocol steps.

e After completing all steps, click **Continue** on the VWorks prompt to continue the automation protocol. Do not wait for the conclusion of the thermal cycler ligation program to continue the protocol.

14 Once the ligation program in Table 30 is finished and you are prompted by VWorks, transfer the plate from the thermal cycler to Bravo deck position 4. Carefully unseal the plate, then click **Continue** on the VWorks prompt to resume the Capture_v1.1.pro protocol.



15 When prompted by VWorks as shown below, remove and discard the SSC Buffer plate from position 3 of the Bravo deck. Place the 50 mM NaOH source plate at position 3 for use in the elution steps of the protocol. After carefully unsealing the source plate, click **Continue** on the prompt to resume the protocol.

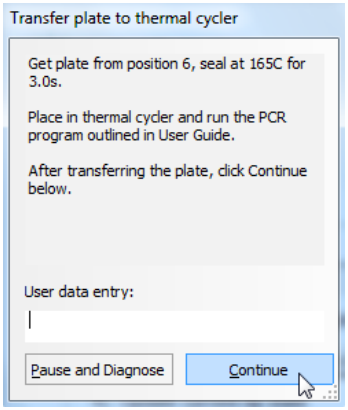


The NGS Bravo completes the liquid-handling steps for elution of the captured target DNA followed by preparation of PCR reactions for amplification.

NOTE

To expedite thermal cycler warm-up for the subsequent PCR program on page 63, 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 16.

16 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 31, using a heated lid.

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

Table 31 HaloPlex post-capture DNA amplification PCR program

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

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 64. 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 4. 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 surface decontamination wipe.
2. Let the AMPure XP beads come to room temperature for at least 30 minutes.

   Do not freeze the AMPure XP beads at any time.
3. Mix the room-temperature bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Prepare a Nunc DeepWell source plate containing AMPure XP beads. For each well to be processed, add 100 µL of homogeneous AMPure XP beads per well to the Nunc DeepWell plate.
5. Place a red insert on Bravo deck position 6.
6. 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.
7. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
8. Prepare a Thermo Scientific reservoir containing 15 mL of the final sample elution buffer [nuclease-free 10 mM Tris-acetate or Tris-HCl buffer (pH 8.0)].
9. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Bravo and Run the Purification_v1.1.pro VWorks Protocol

1. On the VWorks HaloPlex form, under **Step**, select **04 Purification_v1.1.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 32.

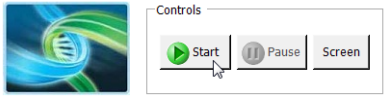
Table 32 Initial Bravo deck configuration for Purification_v1.1.pro

Location	Content
1	Empty Axygen 96 Deep Well Plate (square wells) for waste
2	New tip box
3	Empty full-skirted 96-well Eppendorf twin.tec plate
4	—(empty)—
5	AMPure XP beads 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	Nuclease-free water 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.

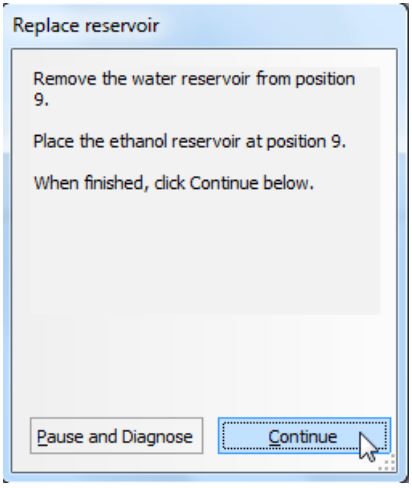
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 21 for more information on using this segment of the form during the run.

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



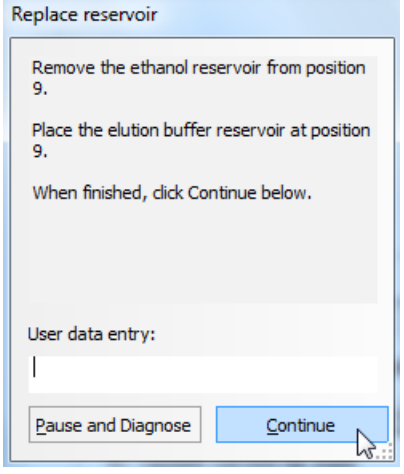
8 When prompted by VWorks as shown below, remove the water reservoir from position 9 of the Bravo deck and replace it with the 70% ethanol reservoir.

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



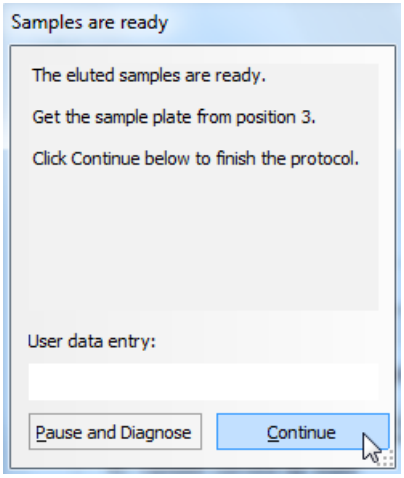
9 When prompted by VWorks as shown below, remove the 70% ethanol reservoir from position 9 of the Bravo deck and replace it with the final sample elution buffer reservoir.

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



The NGS Bravo completes the liquid-handling steps for elution of the captured target DNA.

10 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 5. 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 69) or 2100 Bioanalyzer (see page 70).

Enriched library samples may also be qualitatively analyzed using gel electrophoresis. Sample gel electrophoresis results are provided in the Reference section on page 81.

Expected Results

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

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

The amplicons should range from 175 to 625 bp in length, with the majority of products sized 225 to 525 bp. Amplicons in the 175 to 625 bp size range should be included for quantitation of the enriched target DNA in each sample. Any spurious DNA products outside of this size range in any sample should be excluded from the target DNA quantitation results.

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.

2 Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 4200 TapeStation and start the run.

3 Analyze the electropherogram for each sample according to the analysis guidelines on page 71.

See Figure 8 for a sample electropherogram.

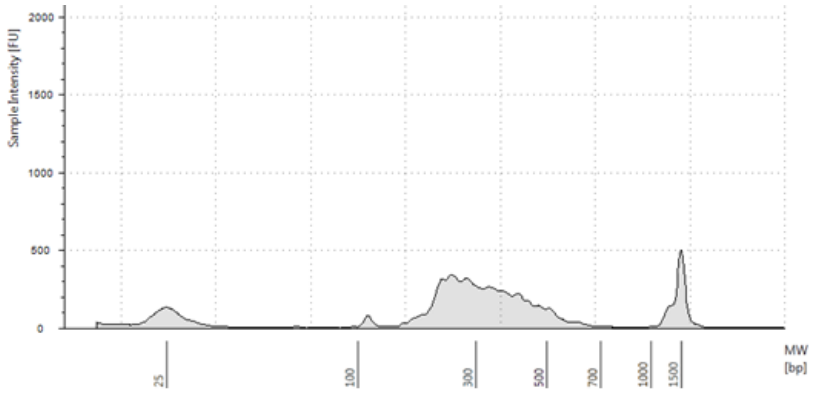


Figure 8 Validation of HaloPlex enrichment by TapeStation analysis.

Option 2: Analysis using the 2100 Bioanalyzer System

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 instrument and start the run within five minutes after preparation.

3 Analyze the electropherogram for each sample according to the analysis guidelines on page 71.

See Figure 9 for a sample 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.

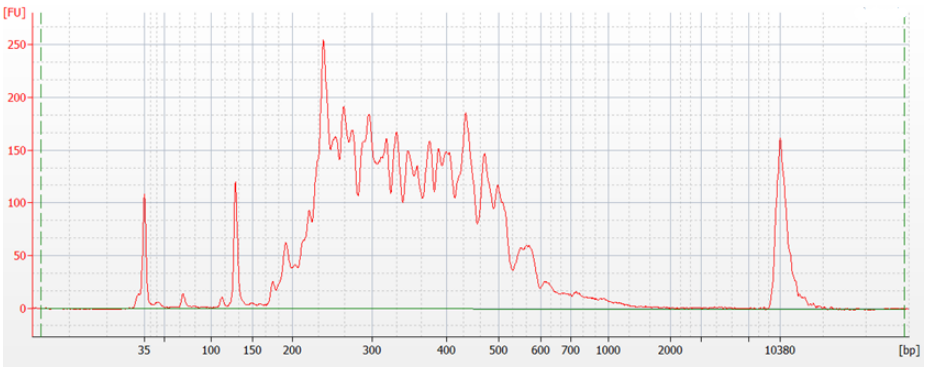


Figure 9 Validation of HaloPlex enrichment by Agilent 2100 Bioanalyzer system analysis.

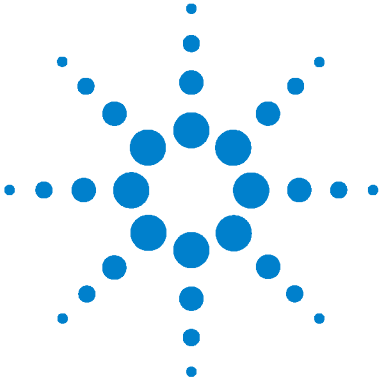
Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 225 to 525 bp.
- Determine the concentration of enriched target DNA in the sample by integration under the peak between 175 and 625 bp. Peaks at <150 bp may be observed, but should be excluded from quantitation.
- Some designs may generate a peak at about 125 bp. This peak is associated with an adaptor-dimer product which will cluster and generate sequence that does not map to the genome. If the molar fraction of the 125 bp peak is greater than 10%, do another round of AMPure purification after pooling samples. First, pool equimolar amounts of libraries to be multiplexed, using concentrations determined for the 175–625 peak of each sample. Using 40 µL of the pooled libraries, purify the DNA using AMPure XP beads according to the protocol starting on page 73.

Step 6. 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. 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 Index 1 (i7) read.
- For complete HaloPlex index sequence information see page 80.
- Before aligning reads to the reference genome, trim the Illumina adaptor sequences from the reads.



4 Appendix: 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 125 bp size. Only do this protocol if electrophoretic analysis of the target-enriched library samples shows a peak at approximately 125 bp which represents a molar fraction of >10% of DNA in the sample (see page 69 to page 71.)

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 tubes with open lids at room temperature until the residual ethanol completely evaporates.

Make sure all ethanol has evaporated before continuing.

15 Remove tubes from the magnetic device and add 40 µL of 10 mM Tris-acetate or Tris-HCl buffer (pH 8.0) to each sample.

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

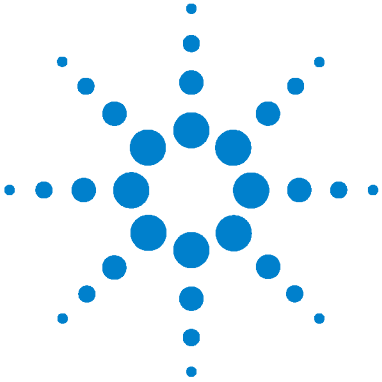
16 Mix thoroughly by pipetting up and down 15 times using a 100-µL pipette set to 30 µL.

17 Incubate for 2 minutes at room temperature to allow elution of DNA.

18 Put the tube in the magnetic 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.



5 Reference

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

Kit Contents

Component kits supplied with the HaloPlex Target Enrichment System are listed in Table 33. Reagents included in the HaloPlex Target Enrichment System-ILM Box 1 are listed in Table 34 on page 78.

Table 33 HaloPlex Target Enrichment System Kit Contents-Custom Designs

Design Type	Kit Part Number	HaloPlex Target Enrichment System-ILM, Box 1	HaloPlex Magnetic Beads Box 2
		Store at –20°C	Store at +4°C
HaloPlex 1-500 kb with <15,000 Probes, ILM, 96 Reactions	G9901B	5190-8050 **OR** 5190-8051*	5190-5386
HaloPlex 1-500 kb with 15,000-20,000 Probes, ILM, 96 Reactions	G9961B	5191-4068 **OR** 5191-4069‡	5190-5386
HaloPlex 0.5-2.5 Mb OR <0.5 Mb with >20,000 probes, ILM, 96 Reactions	G9911B	5190-8052 **OR** 5190-8053†	5190-5386
HaloPlex 2.6 Mb-5 Mb, ILM, 96 Reactions	G9921B	5190-8054 **OR** 5190-8055†	5190-5386

* Part number 5190-8050, 5191-4068, 5190-8052, or 5190-8054 is provided for the first order of a specific HaloPlex Probe design. Re-order kits, containing previously-purchased HaloPlex Probe designs, include Box 1 part number 5190-8051, 5191-4069, 5190-8053, or 5190-8055.

Table 34 HaloPlex Target Enrichment System Box 1 Contents

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

* 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 35 for a plate map.

Table 35 Plate map for HaloPlex Indexing Primers A01 through H12 provided in blue plate

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

Nucleotide Sequences of HaloPlex Indexes

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

Table 36 HaloPlex Indexes, for indexing primers provided in blue 96-well plate

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

Qualitative analysis of enrichment by gel electrophoresis

Enrichment products may be qualitatively analyzed by gel electrophoresis. Analyze 5 µL of each enriched library sample (enriched ECD sample or experimental enriched libraries) by electrophoresis on a Novex 6% polyacrylamide TBE pre-cast gel. See page 43 for additional gel analysis protocol recommendations.

Successful enrichment is indicated by the presence of a smear of amplicons from approximately 225 to 525 bp in each enrichment library lane. For some probe designs, low molecular weight (<150 bp) bands may also be visible, but should not be included in enriched sample quantitation. See Figure 10 for a sample gel analysis image.

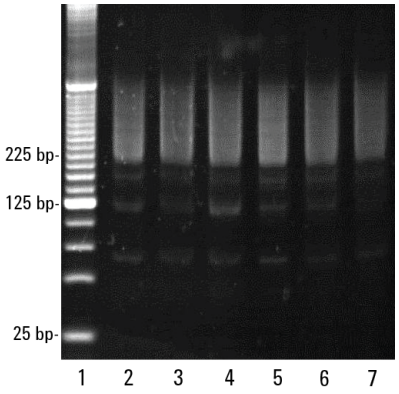


Figure 10 Validation of HaloPlex enrichment process by gel electrophoresis. Lane 1: 25-bp DNA ladder, Lanes 2-7: enriched library samples.

In This Book

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

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