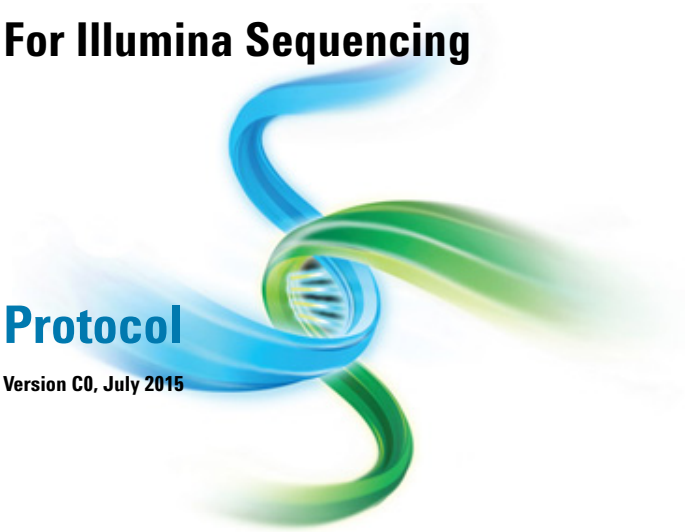
# HaloPlex Exome Target Enrichment System

## Automation Protocol

## For Illumina Sequencing



## Protocol

Version C0, July 2015

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Agilent Technologies

# Notices



## Manual Part Number

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5301 Stevens Creek Blvd
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# In this Guide...

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

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

### 3 Sample Preparation

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

### 4 Reference

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

## What's New in Version C.0

- Updated product labeling statement.

## What's New in Version B.0

- Support for kits supplied with either of two indexing primer configurations.

  Kits with revised index configuration (typically received December, 2014 or later) include indexing primers A01 through H12 provided in a blue plate. For kit content details see page 92. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 39 on page 95.

  Kits with original index configuration (typically received before December, 2014), include indexing primers 1–96 provided in a clear plate. For kit content details see page 96. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 43 on page 99 through Table 48 on page 104.

  Protocol steps for indexing using primers provided in either configuration are identical.
- Updated ordering information for Agilent 2200 TapeStation consumables (Table 3 on page 11)
- Instructions for obtaining Agilent's SureCall analysis software (page 89)

# Content

## 4 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 protocols in this manual are for use with Agilent's G9906D HaloPlex Exome Target Enrichment System for automation. The protocol is not compatible with other versions of HaloPlex reagent kits, including custom kits, Cardiomyopathy Research Panel kits, and Cancer Research Panel kits.
- 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.
- 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 Exome Target Enrichment

| Description | Vendor and part number |
|---|---|
| HaloPlex Exome Target Enrichment System Kit, ILM, 96 reactions* | Agilent, p/n G9906D |
| Herculase II Fusion Enzyme with dNTPs (100 mM; 25 mM for each nucleotide), 200 reactions | Agilent p/n 600677 |
| Nuclease-free Water (not DEPC-treated) | Ambion Cat #AM9930 |
| Agencourt AMPure XP Kit<br>5 mL<br>60 mL<br>450 mL | Beckman Coulter Genomics<br>p/n A63880<br>p/n A63881<br>p/n A63882 |
| 10 M NaOH, molecular biology grade | Sigma, p/n 72068 |
| 2 M acetic acid | Sigma, p/n A8976 |
| 10 mM Tris-HCl, pH 8.0 or 10 mM Tris-acetate, pH 8.0 | General laboratory supplier |
| 100% Ethanol, molecular biology grade | Sigma-Aldrich p/n E7023 |
| Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer | |
| 100 assays, 2-1000 ng<br>500 assays, 2-1000 ng | Life Technologies p/n Q32850<br>Life Technologies p/n Q32853 |

* 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 2** Required Equipment for HaloPlex Exome Target Enrichment Automated Protocols

| Description | Vendor and part number |
|---|---|
| Agilent NGS Bravo Option A* with VWorks software version 11.3.0.1195 | Contact Agilent Automation Solutions for ordering 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 | Agilent SureCycler 8800, p/n G8800A, 96 well plate module, p/n G8810A, and 384 well plate module, p/n G8820A, or equivalent thermal cycler† and accessories |
| Eppendorf twin.tec full-skirted 96-well PCR plates | Eppendorf p/n 951020401 or 951020619 |
| Eppendorf twin.tec full-skirted 384-well PCR plates* | Eppendorf p/n 951020702 |
| Eppendorf twin.tec half-skirted 96-well PCR plates‡ | Eppendorf p/n 951020303 |
| Thermo Scientific Reservoirs | Thermo Scientific p/n 1064156 |
| Nunc DeepWell Plates, sterile, 1.3-mL well volume | Thermo Scientific p/n 260251 |
| Axygen 96 Deep Well Plate, 2.2 mL, Square Well (waste reservoirs) | Axygen p/n P-2ML-SQ-C<br>E & K Scientific p/n EK-2440 |
| Magnetic separator **<br>1.5 mL tube-compatible separator or<br>Conical vial-compatible separator | Life Technologies<br>DynaMag-2 magnet, p/n 12321D or equivalent<br>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 |
| NucleoClean Decontamination Wipes | Millipore p/n 3097 |
| Qubit 2.0 Fluorometer | Life Technologies p/n Q32866 |
| Qubit assay tubes | Life Technologies p/n Q32856 |
| Vortex mixer | General laboratory supplier |

* Protocols are also compatible with Agilent NGS Workstation Option B. See page 12 for more information.

† Thermal cycler must be compatible with 0.2 mL tubes.

‡ Compatible with Agilent SureCycler 8800.

** Select the appropriate device based on run size. See page 56 for the magnetic bead volume required for your run size.

# Optional Validation Reagents and Equipment

**Table 3** Reagents and Equipment for Optional Validation Methods

| Description | Vendor and part number |
|---|---|
| 2200 TapeStation Platform and Consumables | |
| 2200 TapeStation | Agilent p/n G2964AA or G2965AA |
| High Sensitivity D1000 ScreenTape | Agilent p/n 5067-5584 |
| High Sensitivity D1000 Reagents | Agilent p/n 5067-5585 |
| 2100 Bioanalyzer Platform and Consumables | |
| 2100 Bioanalyzer Laptop Bundle | Agilent p/n G2943CA |
| 2100 Bioanalyzer Electrophoresis Set | Agilent p/n G2947CA |
| High Sensitivity DNA Kit | Agilent p/n 5067-4626 |
| Gel Electrophoresis Platform and Consumables | |
| XCell SureLock Mini-cell | Life Technologies p/n EI0001 |
| Novex 6% Polyacrylamide, TBE Pre-cast Gels | Life Technologies p/n EC62655BOX |
| Novex TBE Running Buffer, 5X | Life Technologies p/n LC6675 |
| Novex High-density TBE Sample Buffer, 5X | Life Technologies p/n LC6678 |
| GelRed Nucleic Acid Stain, 3X in water | Biotium p/n 41001 |
| DNA molecular weight markers | General laboratory supplier |
| UV-transilluminator | General laboratory supplier |

# 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 4 below, and verify that the listed adapters are available for your workstation.

**Table 4** 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 Exome Target Enrichment



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

# About the Agilent NGS Bravo Option A

## 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 seven interchangeable fixed-tip or disposable-tip pipette heads, it accurately dispenses fluids from 0.1 µL to 250 µL.

**CAUTION** Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using your Bravo platform. Refer to the *Bravo Platform User Guide* (G5409-90006) and the *VWorks Software User Guide* (G5415-90063).

### 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.

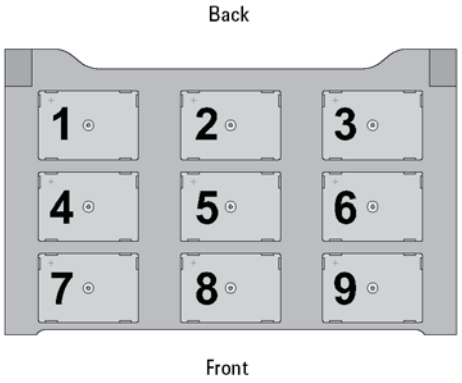



**Figure 1** Bravo platform deck

### Setting the Temperature of Bravo Deck Heat Blocks

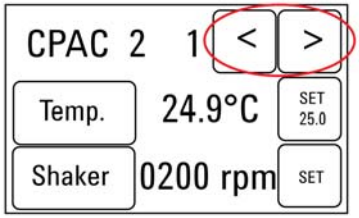
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include low-temperature (4°C) or high-temperature (54°C) incubation steps may be expedited by pre-setting the temperature of the affected block before starting 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 5 for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

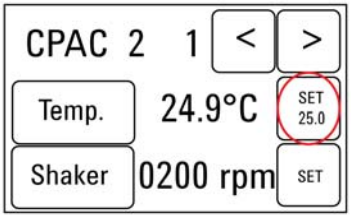
**Table 5** Inheco Multi TEC Control touchscreen designations

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

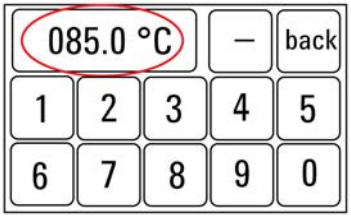
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



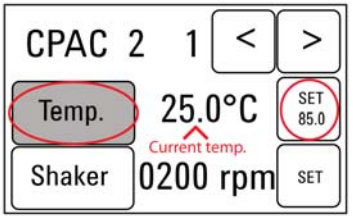
2 To set the temperature of the selected block, press the SET button.




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 PC supplied with 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 is provided below. Each time a specific VWorks protocol is used in the HaloPlex workflow, any settings required for that protocol are included in the relevant section of this manual.

**NOTE**

The instructions in this manual are compatible with VWorks software version 11.3.0.1195.

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_Exome.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.)

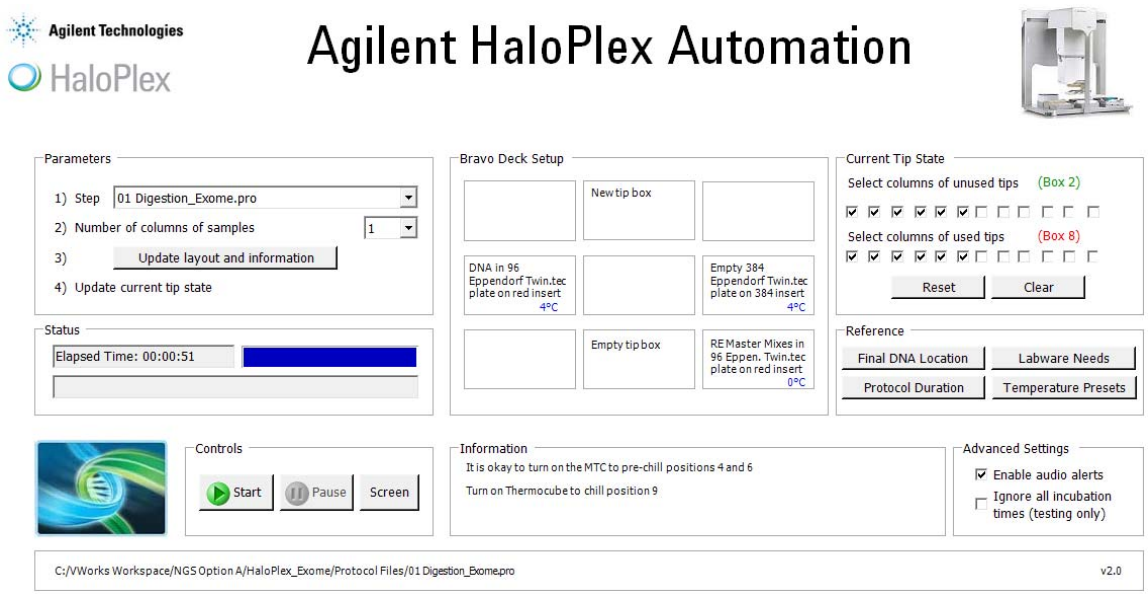
### Using the HaloPlex_Exome.VWForm to setup and start a run

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

1. Open the form using the HaloPlex_Exome.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.**

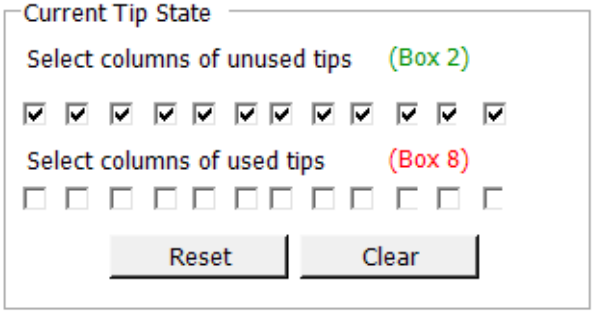
**NOTE** The displayed protocol will not run unless the **Update layout and information** button has been clicked.

4. The **Bravo Deck Setup** region of the form will then display the required placement of reaction components and labware on the 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.

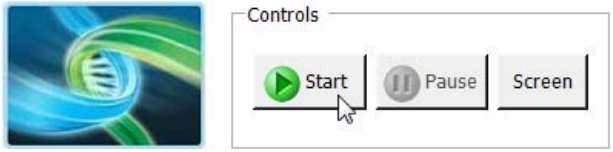
For an empty tip box, all positions of the **Current Tip State** used tip indicator (bottom portion, Box 8) should be cleared, as shown above. Clicking **Reset** clears all columns for position 8.

**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.

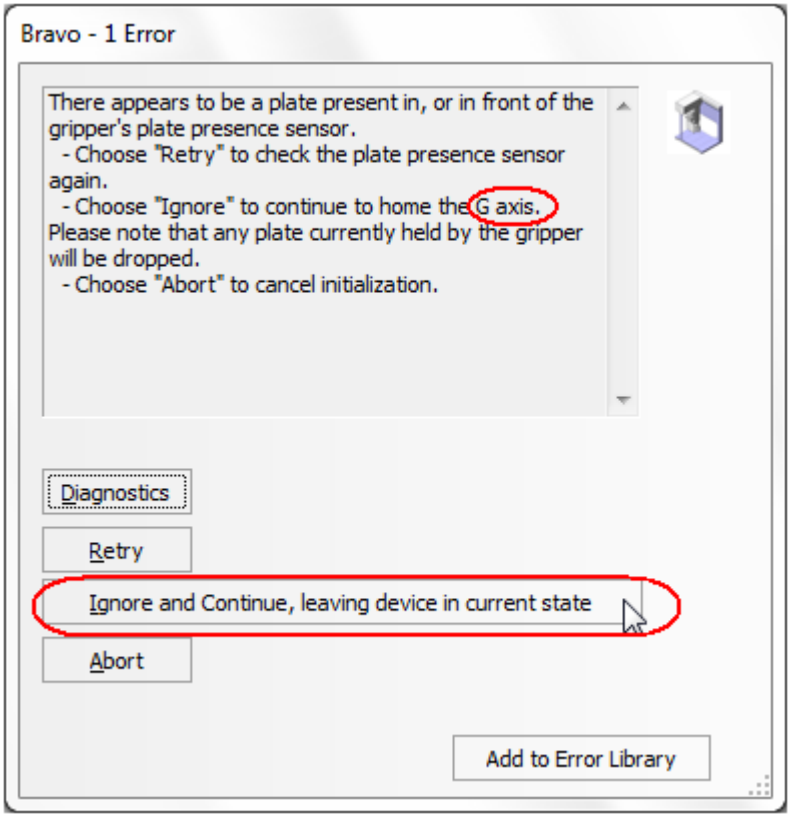
6 After verifying that the NGS Bravo has been set up correctly, click **Start** in the **Controls** section of the form to begin the run. Do not use the Start button on the VWorks Control Toolbar; runs must be initiated using the start button on the HaloPlex.VW Form, shown below.



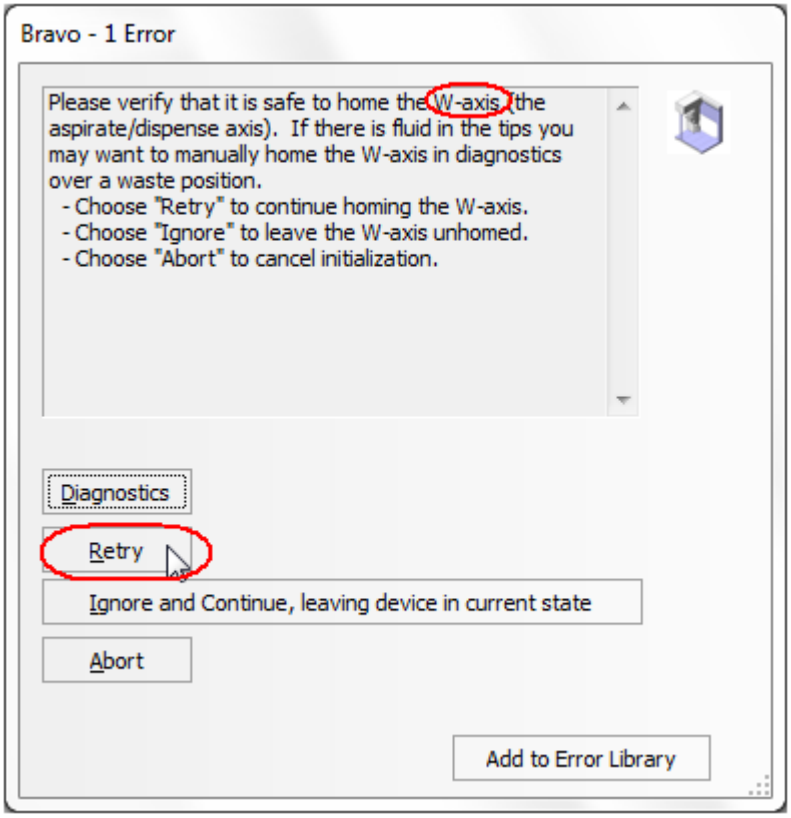
### Error messages encountered at start of run

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**.



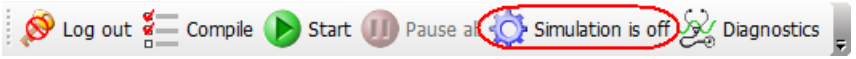
**2** If you encounter the W-axis error message shown below, select **Retry.**



### 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 Exome Target Enrichment Procedure

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

Table 6 summarizes how the VWorks automation protocols are integrated into the HaloPlex Exome workflow. See the Sample Preparation chapter for complete instructions for use of the VWorks protocols for sample processing.

**Table 6** Overview of VWorks protocols used during the workflow

| Workflow Step | VWorks Protocol used for Automation |
|---|---|
| Digest genomic DNA in eight separate Restriction Enzyme Master Mixes A to H | Digestion_Exome.pro |
| Hybridize restriction digests A to H to HaloPlex probes A to H and index DNA | Hybridization_Exome.pro |
| Pool hybridization reactions for each DNA sample | Post Hybridization Pooling_Exome.pro |
| Capture the biotin-tagged hybrids on streptavidin-coated magnetic beads and amplify the enriched DNA | Capture_Exome.pro |
| Purify amplified libraries | Purification_01_Exome.pro, then Purification_02_Exome.pro |

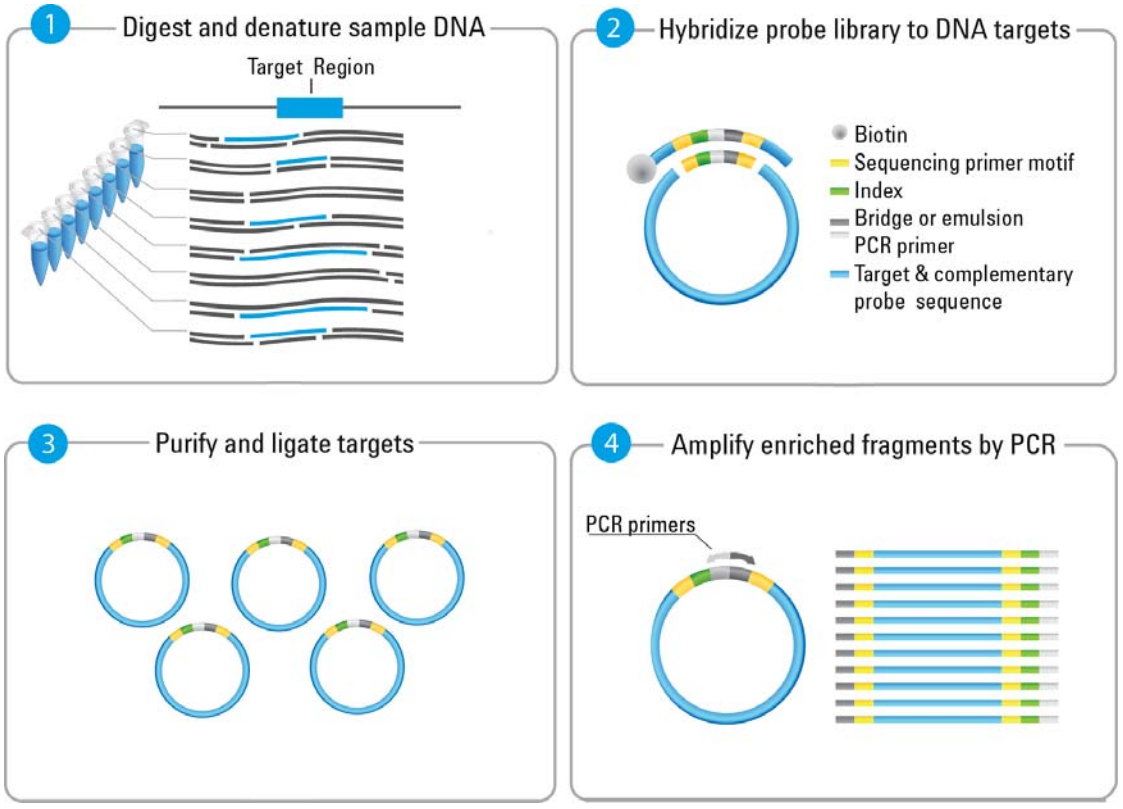



**Figure 2** Overall HaloPlex Exome target-enriched sequencing sample preparation workflow.

# 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 7** 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 92) 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 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 probe (see Figure 2), you will need to prepare a separate plate containing the HaloPlex Indexing Primer Cassettes. Assign the wells to be indexed with their respective indexing primers during experimental design. See the Reference chapter for the nucleotide sequences of the 96 indexes used in the HaloPlex Exome Target Enrichment System.

**CAUTION**

This guide includes information for kits containing two different sets of indexing primers. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Kits with indexing primers supplied in a blue plate include 8-bp indexes A01 through H12. See page 94 through page 95 for indexing primer A01–H12 plate map and nucleotide sequence information.

Kits with indexing primers supplied in a clear plate include 8-bp indexes 1 through 96. See page 98 through page 104 for indexing primer 1–96 plate map and nucleotide sequence information.

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

## 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 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

The hybridization time for HaloPlex Exome enrichment is 16 to 24 hours. Based on the desired start and end time of the hybridization incubation, calculate the appropriate start time for the DNA digestion protocol, using the automation run time estimates provided in a link from the HaloPlex Exome Form in VWorks.

# 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 exome target-enriched, indexed library is prepared.

The HaloPlex Exome 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 multiple 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 no. G9900-90050, available at http://www.genomics.agilent.com. This publication provides a PCR-based protocol for assessment of DNA integrity and provides HaloPlex protocol modifications for improved performance from lower-quality DNA samples.

### Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a NucleoClean 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 36, 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 36. 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 the 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 Mix Source Plate

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

**Table 8** Preparation of RE Buffer + BSA mixture for Digestion_Exome.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 |
| BSA 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** |

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 + BSA 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 9** Preparation of RE Master Mixes A–H for Digestion_Exome.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 + BSA | 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 10 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 10** Preparation of the RE Master Mix Source Plate for Digestion_Exome.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 |

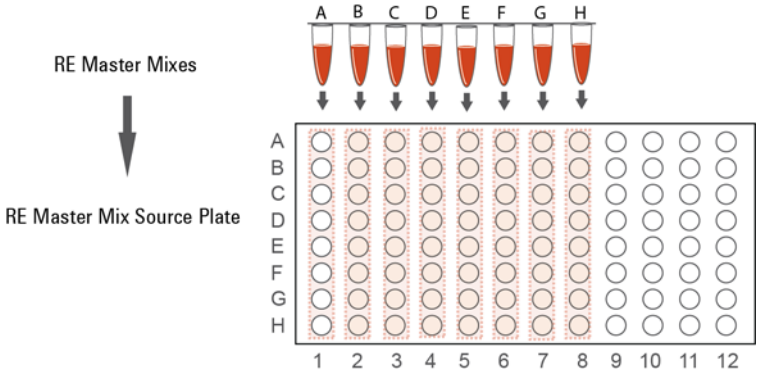


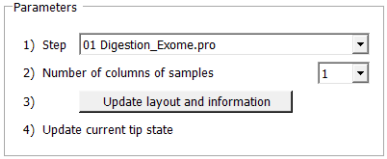

**Figure 3** Preparation of the RE Master Mix source plate for automation protocol Digestion_Exome.pro.

### Load the NGS Bravo and Run the Digestion_Exome.pro VWorks Protocol

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

2 Log in to the VWorks software.

3 On the setup form, under **Step**, select **01 Digestion_Exome.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 11.

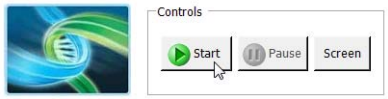
**Table 11** Initial Bravo deck configuration for Digestion_Exome.pro

| 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)<br>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 19 for more information on using this segment of the form during the run.

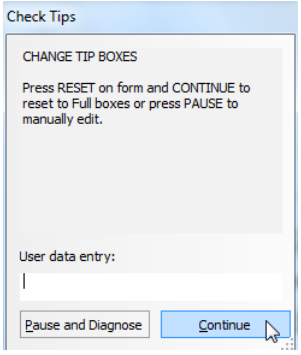
9 When verification is complete, click **Start** to start the run.




**NOTE** If NGS 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 22 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.

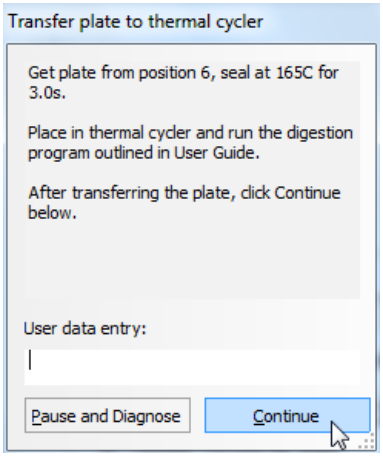
Depending on the run size, you may be prompted to change tip boxes multiple times during the run.




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 12, using a heated lid. After transferring the plate, click **Continue** on the prompt.

**Table 12** Thermal cycler program for HaloPlex 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_Exome.pro protocol and thermal cycler program for plate 2 is finished.

**15** Store the 384-well DNA digestion plate(s) on ice while completing the validation steps below as well as while setting up the subsequent Hybridization protocol.

**16** 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 13 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 13** 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** Add 4 µL nuclease-free water to each ECD digestion reaction well to restore 10 µL/well reaction volumes for the subsequent workflow steps.

**d** Analyze the prepared samples using microfluidic electrophoresis on the 2100 Bioanalyzer (see page 40) or on the 2200 TapeStation (see page 41) or using gel electrophoresis (see page 42).

**NOTE**

After validation of the restiction digest protocol, the ECD sample (from well A1 of the starting 96-well DNA sample plate) is not used for subsequent steps in the workflow.

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 reaction B to be slighltly reduced, compared to the other digestion reactions.

### Option 1: Validation by 2100 Bioanalyzer analysis

Use a High Sensitivity DNA Kit (p/n 5067-4626) and the 2100 Bioanalyzer system with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer system setup instructions.

- Prepare an undigested DNA gel control by combining 0.5 µL of the Enrichment Control DNA stock solution and 3.5 µL of nuclease-free water.
- Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each ECD sample and undigested DNA control for the analysis.
- When loading samples on the chip, load the DNA ladder in the ladder sample well marked on the chip. Load the eight ECD digest samples (A to H) in sample wells 1 to 8, and load the undigested ECD sample in sample well 9. Do not run the undigested ECD control in sample well 1.
- Place the prepared chip into the 2100 Bioanalyzer instrument and start the run within five minutes after preparation.

See Figure 4 for sample Bioanalyzer electrophoresis results.

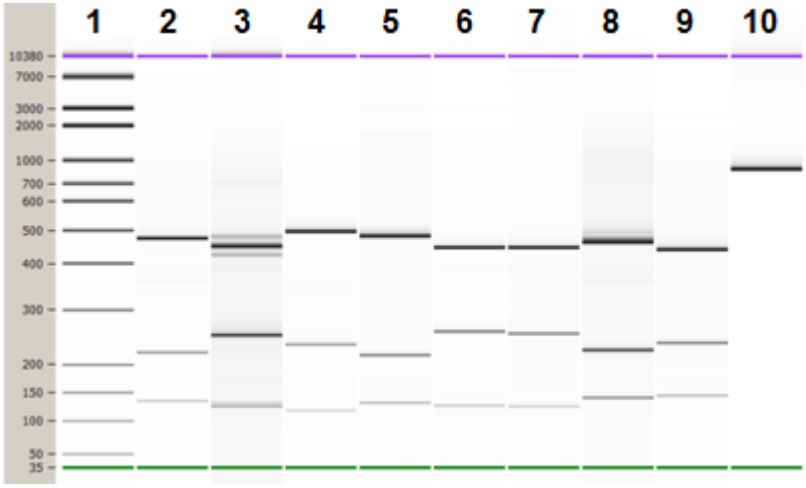



**Figure 4** Validation of restriction digestion by 2100 Bioanalyzer system analysis. Lane 1: 50-bp DNA ladder, Lanes 2-9: ECD digestion reactions A–H, Lane 10: Undigested Enrichment Control DNA.

### Option 2: Validation by 2200 TapeStation analysis

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585). For more information to do this step, see the *2200 TapeStation User Manual*.

- Prepare an undigested DNA gel control by combining 1 µL of the Enrichment Control DNA stock solution and 1 µL of nuclease-free water.
- Prepare the TapeStation samples as instructed in the *2200 TapeStation User Manual*. Use 2 µL of each ECD sample diluted with 2 µL of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.
- Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *2200 TapeStation User Manual*. Start the run.

See Figure 5 for sample TapeStation electrophoresis results.

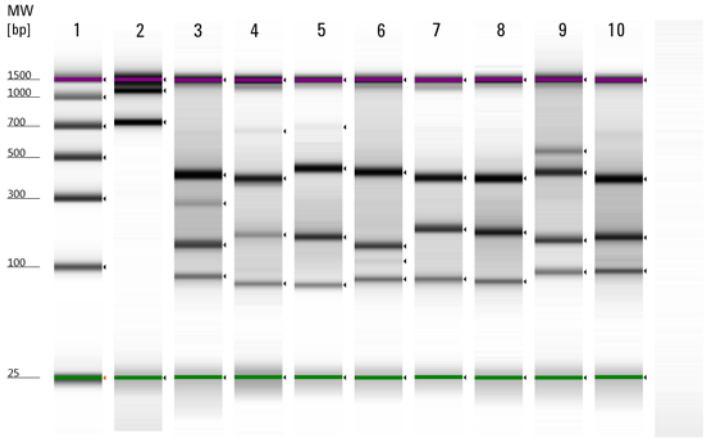



**Figure 5** Validation of restriction digestion by 2200 TapeStation analysis. Lane 1: TapeStation D1000 High-Sensitivity Ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions A–H.

### Option 3: Validation by gel electrophoresis

Use a Novex 6% polyacrylamide TBE pre-cast gel and 1X Novex TBE Running Buffer. For more information to do this step, consult the manufacturer's recommendations.

- Prepare an undigested DNA gel control by combining 2 µL of the Enrichment Control DNA stock solution and 2 µL of nuclease-free water.
- Add 1 µL of Novex Hi-Density TBE Sample Buffer (5X) to each 4-µL ECD sample.
- Load 5 µL of each sample on the gel. In one or more adjacent lanes, load 200 ng of a 50-bp DNA ladder.
- Run the gel at 210 V for approximately 15 minutes.
- Stain the gel in 3X GelRed Nucleic Acid Stain for 10 minutes, and visualize bands under UV radiation.

See Figure 6 for sample gel results.

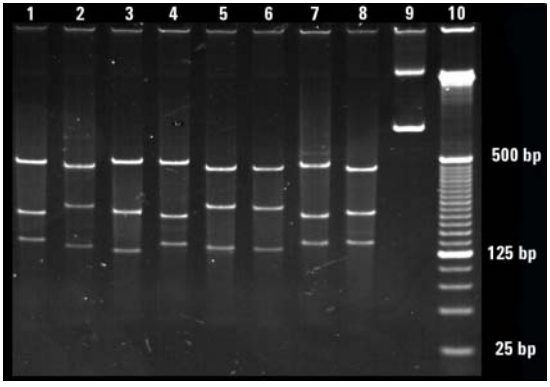



**Figure 6** Validation of restriction digestion by gel electrophoresis. Lanes 1–8: ECD digestion reactions A–H, Lane 9: Undigested Enrichment Control DNA, Lane 10: 25-bp DNA ladder.

**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 72.

# Step 2. Hybridize HaloPlex probes A-H to DNA digests A-H for target enrichment and sample indexing

The HaloPlex Exome probe is provided as eight separate probe solutions in wells A-H of the HaloPlex Probe 8-well Strip.

**CAUTION**

Do **not** pool the probe solutions provided in the HaloPlex Probe 8-well Strip. The eight probe pools must be used for hybridization in separate wells, with the captured DNA being combined after hybridization.

In this step, the eight probe solutions A through H are hybridized, in eight separate hybridization wells, to the DNA restriction fragment libraries A-H prepared in Step 1. The automation protocol below does the liquid handling steps to prepare hybridization reactions in which digests A-H are matched with corresponding probes A-H. (Sample digest A is hybridized to Probe A, while sample digest B is hybridized to Probe B, and so on.)

Hybridization reactions are set up to include the appropriate indexing primers. During the hybridization reaction Illumina sequencing motifs, including index sequences, are integrated into the targeted fragments. For sample indexing primer assignments, see the Reference chapter for the nucleotide sequences of the 96 indexes used in the HaloPlex Exome Target Enrichment System.

**CAUTION**

This guide includes information for kits containing two different sets of indexing primers. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Kits with indexing primers supplied in a blue plate include 8-bp indexes A01 through H12. See page 94 through page 95 for indexing primer A01–H12 plate map and nucleotide sequence information.

Kits with indexing primers supplied in a clear plate include 8-bp indexes 1 through 96. See page 98 through page 104 for indexing primer 1–96 plate map and nucleotide sequence information.

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

For 12-column runs, restriction digests from each of the two 384-well plates prepared in Step 1 will be processed during separate segments of the Hybridization_Exome.pro protocol. Begin the protocol with the first 384-well restriction digest plate on the Bravo deck. The VWorks software will prompt you to replace the first 384-well restriction digest plate with the second restriction digest plate at the appropriate time.

### Prepare the NGS Bravo

1. Gently wipe down the Bravo deck with a NucleoClean decontamination wipe.
2. Place a red insert on Bravo deck position 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 positions 4 and 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 hybridization reaction on page 50, 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 for hybridization. Be sure that the 96-well block is in the thermal cycler before initiating the program for warm-up.

### Prepare the Indexing Primer Cassette Source Plate

**CAUTION**

This guide includes information for kits containing two different sets of indexing primers. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Reference information for kits with indexing primers supplied in a blue plate begins on page 92.

Reference information for kits with indexing primers supplied in a clear plate begins on page 96.

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

The Indexing Primer Cassette solutions supplied with the kit must be diluted for use in the exome automation protocol as described below. The well position for each index should correspond to the position of the DNA sample assigned to that index in the original, 96-well pre-digestion DNA sample plate.

1 In a full-skirted 96-well Eppendorf twin.tec plate, dispense 22.5 µL of nuclease-free water in each sample well used in the run.

2 Dispense 27.5 µL of the appropriate Indexing Primer Cassette to each sample well position.

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

3 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec. Vortex the plate to mix the index dilutions, then briefly spin to collect the liquid. Keep the Indexing Primer Cassette source plate on ice.

### Prepare the Hybridization Master Mix Source Plate

1 Obtain the black-marked Exome Probe Strip from Box 1. Label the black-marked tube with A, then continue labeling the remaining tubes with B through H, in order. Keep the probe strip tube on ice.

**CAUTION** It is important to use the probe strip in the proper orientation when preparing the Hybridization Master Mixes as described below. The black color marker on the tube strip and cap strip are used to mark tube A.

2 To prepare the eight Hybridization Master Mixes, label eight 1.5-mL tubes with A through H. Dispense the appropriate volume of Hybridization Solution for your run size, shown in Table 14, to each of the eight tubes.

**Table 14** Hybridization Solution volumes for Hybridization Master Mix preparation

| 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 |
|---|---|---|---|---|---|---|---|
| Hybridization Solution | 6.25 µL | 79.7 µL | 132.8 µL | 185.9 µL | 239.1 µL | 345.3 µL | 690.6 µL |

3 Add the appropriate volume of probe solution from the HaloPlex Probe Strip to the corresponding Hybridization Master Mix tube. Add probe from tube A of the probe strip only to Hybridization Master Mix tube A. Repeat the process for probes B through H, adding each probe solution to a single master mix tube.

**Table 15** HaloPlex Probe volumes for Hybridization Master Mix preparation

| 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 A, B, C, D, E, F, G, **OR** H from provided strip tube | 1 µL | 12.8 µL | 21.3 µL | 29.8 µL | 38.3 µL | 55.3 µL | 110.5 µL |

4 Aliquot the Hybridization Master Mixes A through H to a full-skirted 96-well Eppendorf twin.tec plate as shown in Figure 7. Add the volumes indicated in Table 16 of each master mix to each well of the indicated column of the twin.tec plate. Keep the master mixes on ice during the aliquoting steps.

**Table 16** Preparation of the Hybridization Master Mix Source Plate for Hybridization_Exome.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 |
| Hyb Master Mix A | Column 1 (A1-H1) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix B | Column 2 (A2-H2) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix C | Column 3 (A3-H3) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix D | Column 4 (A4-H4) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix E | Column 5 (A5-H5) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix F | Column 6 (A6-H6) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix G | Column 7 (A7-H7) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |
| Hyb Master Mix H | Column 8 (A8-H8) | 10.6 µL | 18.4 µL | 26.1 µL | 33.8 µL | 49.2 µL | 99.2 µL |

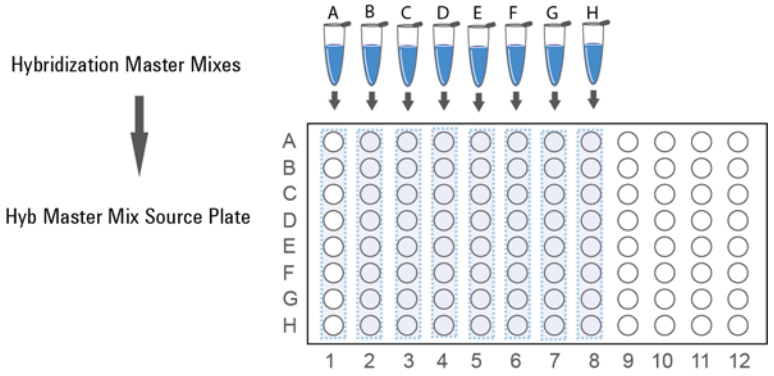



**Figure 7** Preparation of the Hybridization Master Mix source plate for automation protocol Hybridization_Exome.pro.

### Load the NGS Bravo and Run the Hybridization_Exome.pro VWorks Protocol

1 On the VWorks HaloPlex form, under **Step**, select **02 Hybridization_Exome.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 17.

**Table 17** Initial Bravo deck configuration for Hybridization_Exome.pro

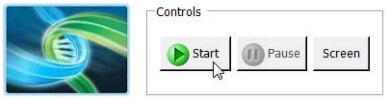
| Location | Content |
|---|---|
| 1 | —(empty)— |
| 2 | New tip box |
| 3 | For 12-column runs only: Empty 384-well Eppendorf twin.tec plate (no 384-well insert required)<br>For 1- to 6-column runs: empty |
| 4 | Digested DNA in 384-well plate, seated on 384-well insert* |
| 5 | —(empty)— |
| 6 | Empty 384-well Eppendorf twin.tec plate, seated on 384-well insert |
| 7 | —(empty)— |
| 8 | Empty tip box |
| 9 | Hybridization Master Mix source plate (96-well Eppendorf twin.tec plate) seated on red insert |

* For 12-column runs, begin the protocol with the first digested DNA sample plate at position 4, and keep the second digested DNA sample plate sealed on ice until you are prompted to add it to the NGS Bravo during the run.

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 19 for more information on using this segment of the form during the run.

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

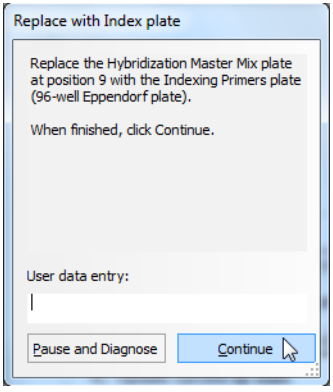



For each DNA sample, the NGS Bravo combines the completed RE digestion reaction A-H with the corresponding Hybridization Master Mix A-H in wells of a 384-well plate.

The remaining steps of the **Hybridization_Exome.pro** automation protocol differ for runs of 1-6 columns of samples (where a single 384-well digested DNA sample plate is processed) versus runs of 12 columns (where two 384-well digested DNA sample plates are processed). For 1-6 column runs, follow the steps immediately below. For 12-column runs, go to page 51.

### Hybridization Workflow for 1-6 Column Runs

1 When the NGS Bravo has finished combining the DNA digests and Hybridization master mixes, you will be prompted by VWorks as shown below.

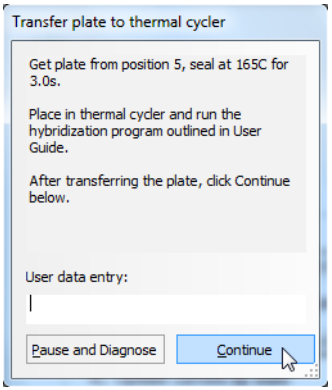



**a** Remove and discard the Hybridization Master Mix source plate from position 9.

**b** Obtain the Indexing Primer Cassette source plate (prepared on page 45), carefully unseal the plate to prevent splashing, and load the plate on position 9, seated on the red insert.

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

2 When the NGS Bravo has finished adding the indexing primers to the hybridization reactions, you will be prompted by VWorks as shown below.




**a** Remove the hybridization plate from position 5. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec. Spin the plate briefly.

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

**c** Place the plate in the thermal cycler and run the hybridization program in Table 18. Use a heated lid. Do **not** include a low-temperature hold step in the thermal cycler program. Incubation at 54°C for greater than 24 hours is not recommended.

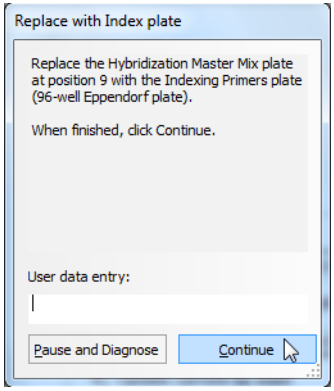
**Table 18** Thermal cycler program for HaloPlex Exome probe hybridization

| Step | Temperature | Time |
|---|---|---|
| Step 1 | 95°C | 10 minutes |
| Step 2 | 54°C | 16-24 hours |

**d** After the 16-24 hour incubation step, proceed to "Step 3. Pool hybridized DNA samples" on page 55.

### Hybridization Workflow for 12 Column Runs

1 When the NGS Bravo has finished combining the DNA digests and Hybridization master mixes for the first plate of digested DNA samples, you will be prompted by VWorks as shown below.

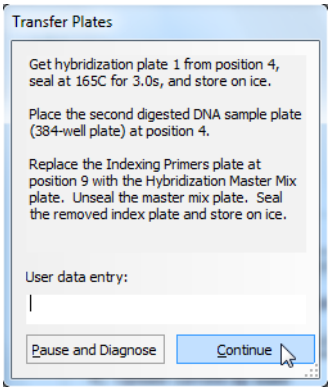



**a** Remove the Hybridization Master Mix source plate from position 9. Seal the master mix plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec, and store the plate on ice.

**b** Obtain the Indexing Primer source plate (prepared on page 45), carefully unseal the plate to prevent splashing, and load the plate on position 9, seated on the red insert.

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

2 When the NGS Bravo has finished adding the indexing primers to the first plate of hybridization reactions, you will be prompted by VWorks as shown below.




**a** Remove the hybridization plate 1 from position 4. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec. Place hybridization plate 1 on ice while the NGS Bravo prepares the second hybridization plate.

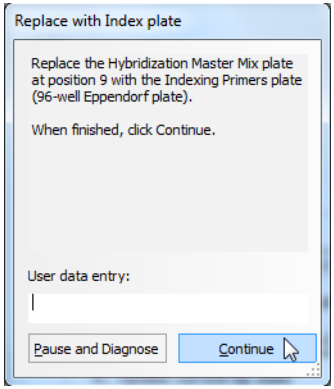
**b** Place the second digested DNA sample plate at position 4, seated on the 384-well plate insert. Carefully unseal the plate.

**c** Remove the Indexing Primer source plate from position 9. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec, and store the plate on ice.

**d** Obtain the Hybridization Master Mix source plate from ice, carefully unseal the plate, and load the plate on position 9, seated on the red insert.

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

3 When the NGS Bravo has finished combining the DNA digests and Hybridization master mixes for the second plate of digested DNA samples, you will be prompted by VWorks as shown below.

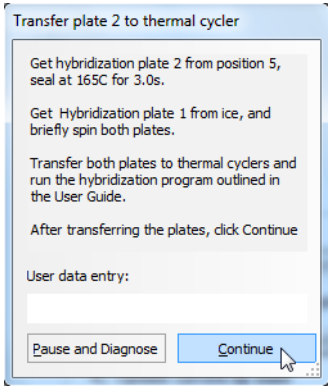



**a** Remove and discard the Hybridization Master Mix source plate from position 9.

**b** Obtain the Indexing Primer source plate from storage on ice, carefully unseal the plate, and load the plate on position 9, seated on the red insert.

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

4 When the NGS Bravo has finished adding the indexing primers to the second plate of hybridization reactions, you will be prompted by VWorks as shown below.




**a** Remove the second hybridization plate from position 5. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

**b** Recover the first hybridization plate from ice storage. Spin both hybridization plates briefly, then place each plate in a thermal cycler and run the hybridization program in Table 19. Use a heated lid. Do **not** include a low-temperature hold step in the thermal cycler program. Incubation at 54°C for greater than 24 hours is not recommended.

**Table 19** Thermal cycler program for HaloPlex Exome probe hybridization

| Step | Temperature | Time |
|---|---|---|
| Step 1 | 95°C | 10 minutes |
| Step 2 | 54°C | 16–24 hours |

**c** When finished transferring the plates, click **Continue** on the VWorks prompt.

**d** After completing the 16-24 hour incubation, proceed to "Step 3. Pool hybridized DNA samples" on page 55.

# Step 3. Pool hybridized DNA samples

In this step, the eight hybridization reactions that correspond to one DNA sample are combined into a single well and mixed with streptavidin beads in preparation for the capture protocol.

The NGS Bravo pools the hybridized DNA samples from the 384-well hybridization plates into wells of a Nunc DeepWell plate in the same sample configuration that was used for the pre-digestion DNA sample plate.

### Prepare the NGS Bravo and reagents

1. Remove reagents to be used in this protocol from cold storage and allow the solutions to reach room temperature:
   - From –20°C storage, remove the Capture Solution.
   - From +4°C storage, remove the HaloPlex Magnetic Beads.
2. Gently wipe down the Bravo deck with a NucleoClean decontamination wipe.
3. For all run sizes, place a 384-well adapter insert on Bravo deck position 6. For 12-column runs only, place a second 384-well adapter insert on Bravo deck position 4.
4. Pre-set the temperature of Bravo deck positions 4 and 6 to 54°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.

### 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 20.

**Table 20** Volume of HaloPlex Magnetic Bead suspension for Post Hybridization Pooling_Exome.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 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 21) to the beads and resuspend by pipetting up and down.

**Table 21** 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 of the pre-digestion DNA sample plate.

### Load the NGS Bravo and Run the Post Hybridization Pooling_Exome.pro VWorks Protocol

1 On the VWorks HaloPlex form, under **Step**, select **03 Post Hybridization Pooling_Exome.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 22.

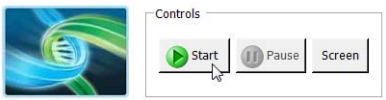
**Table 22** Initial Bravo deck configuration for Post Hybridization Pooling_Exome.pro

| Location | Content |
|---|---|
| 1 | —(empty)— |
| 2 | New tip box |
| 3 | —(empty)— |
| 4 | 384-well adapter insert |
| 5 | HaloPlex magnetic streptavidin bead plate (Nunc DeepWell plate) |
| 6 | 384-well adapter insert |
| 7 | —(empty)— |
| 8 | Empty tip box |
| 9 | —(empty)— |

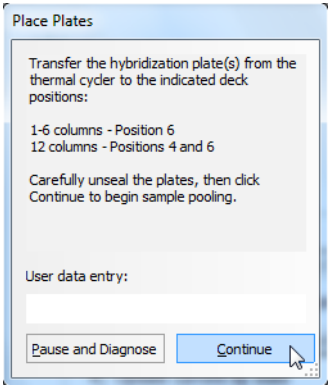
5 Verify that the NGS Bravo has been set up as displayed in the **Bravo Deck Setup** and **Information** regions of the form.

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 19 for more information on using this segment of the form during the run.

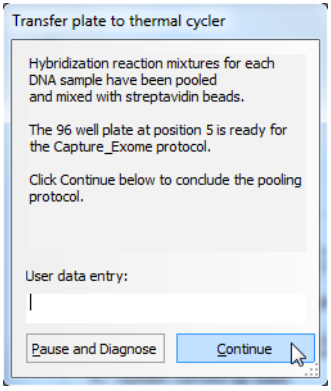
7 When verification is complete, click **Start** to start the run.



8 When prompted by VWorks as shown below, get the hybridization plate(s) from the thermal cycler. Briefly spin the sealed plates to collect the liquid, then load the plate(s) on the indicated position(s) of the Bravo deck. Carefully unseal the plate(s). When finished, Click **Continue** on the prompt to resume the pooling protocol.




The NGS Bravo pools the target DNA-HaloPlex probe hybrids into the appropriate wells of the streptavidin bead-containing Nunc DeepWell plate. When the Bravo has finished the pooling step, you will be prompted by VWorks as shown below.




Remove the sample capture plate from position 5, seal the plate, and store at room temperature while you prepare the components of the Capture_Exome automation protocol. Click **Continue** on the prompt to conclude the pooling protocol.

# Step 4. Capture and amplify the target DNA

In this step, the pooled target DNA-HaloPlex probe hybrids 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 the Wash Solution, Ligation Solution and SSC Buffer from –20°C storage and allow the solutions to reach room temperature.

2 Prepare 50 µL per sample, plus excess, of fresh 50 mM NaOH for use in the DNA elution step on page 60.

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 23** 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 |
|---|---|---|---|---|---|---|
| 60 µL | 540 µL | 1020 µL | 1500 µL | 1980 µL | 2940 µL | 5880 µL |

3 Obtain or prepare 1 µL per sample, plus excess, of 2 M acetic acid, for use in the PCR master mix on page 61.

**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 NucleoClean decontamination wipe.
2. Place a red insert on Bravo deck position 4.
3. Pre-set the temperature of Bravo deck position 4 to 46°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 wash and elution solution source plates

Prepare a separate source plate for each of the solutions listed in Table 24. Use full-skirted 96-well Eppendorf twin.tec plates to prepare all three source plates. For all sample-containing well positions, add the specified volume of solution to each source plate.

**Table 24** Preparation of solution source plates for Capture_Exome.pro protocol

| Solution | Volume to dispense per well of source plate |
|---|---|
| Wash Solution | 110 µL |
| SSC Buffer | 110 µL |
| 50 mM NaOH | 60 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 14 on page 70.

### Prepare the Master Mixes for Capture_Exome.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 25** Preparation of PCR Master Mix for Capture_Exome.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 | 32.2 µL | 410.6 µL | 684.3 µL | 958.0 µL | 1231.7 µL | 1779.1 µL | 3558.1 µL |
| 5X Herculase II Reaction Buffer | 20 µL | 255.0 µL | 425.0 µL | 595.0 µL | 765.0 µL | 1105.0 µL | 2210.0 µL |
| dNTPs (100 mM) | 0.8 µL | 10.2 µL | 17.0 µL | 23.8 µL | 30.6 µL | 44.2 µL | 88.4 µL |
| Primer 1 | 2 µL | 25.5 µL | 42.5 µL | 59.5 µL | 76.5 µL | 110.5 µL | 221.0 µL |
| Primer 2 | 2 µL | 25.5 µL | 42.5 µL | 59.5 µL | 76.5 µL | 110.5 µL | 221.0 µL |
| 2 M Acetic acid | 1 µL | 12.8 µL | 21.3 µL | 29.8 µL | 38.3 µL | 55.3 µL | 110.5 µL |
| Herculase II Fusion DNA Polymerase | 2 µL | 25.5 µL | 42.5 µL | 59.5 µL | 76.5 µL | 110.5 µL | 221.0 µL |
| **Total Volume** | **60 µL** | **765 µL** | **1275 µL** | **1785 µL** | **2295 µL** | **3315 µL** | **6630 µL** |

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 66. The Ligation Master Mix is added to the Master Mix Source Plate just before it is used in the Capture_Exome.pro protocol. Do not add this master mix to the source plate before starting the run.

**Table 26** Preparation of Ligation Master Mix for Capture_Exome.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_Exome.pro

To prepare the Master Mix source plate for Capture_Exome.pro, add the volume indicated in Table 27 of PCR Master Mix to all wells of column 3 of a Nunc DeepWell plate.

**Table 27** Preparation of the Master Mix Source Plate for Capture_Exome.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) | 88.1 µL | 151.9 µL | 215.6 µL | 279.4 µL | 406.9 µL | 821.3 µL |

**NOTE** Columns 1 and 2 of the Master Mix source plate remain empty at this step. You will be prompted to add Ligation Master Mix to Column 2 at the appropriate time during the Capture_Exome.pro protocol. Column 1 will remain empty during the Capture_Exome.pro protocol.

### Load the NGS Bravo and Run the Capture_Exome.pro VWorks Protocol

1 On the VWorks HaloPlex form, under **Step**, select **04 Capture_Exome.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 28.

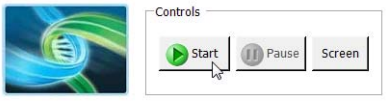
**Table 28** Initial Bravo deck configuration for Capture_Exome.pro

| 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 | Empty half-skirted 96-well Eppendorf twin.tec plate seated on red insert |
| 5 | Pooled hybridized DNA samples + streptavidin beads in Nunc DeepWell plate |
| 6 | Empty half-skirted 96-well Eppendorf twin.tec plate seated on red insert |
| 7 | —(empty)— |
| 8 | Empty tip box |
| 9 | Master Mix source plate (Nunc DeepWell plate) seated on silver insert |

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 19 for more information on using this segment of the form during the run.

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

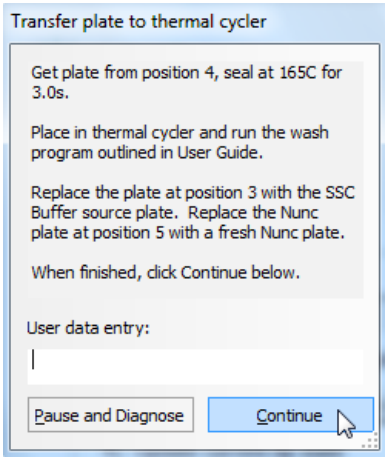


The NGS Bravo completes the liquid-handling steps for capture and washing of the target DNA-HaloPlex probe hybrids on the streptavidin beads.

**NOTE**

To expedite thermal cycler warm-up for the subsequent wash program on page 64, 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 8.

**8** 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 29, using a heated lid.

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

**Table 29** Thermal cycler program for Capture_Exome.pro wash step

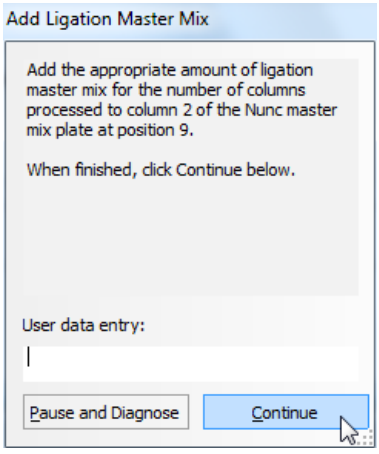
| Step | Temperature | Time |
|---|---|---|
| Step 1 | 46°C | 10 minutes |

**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 used Nunc 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.

9 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.



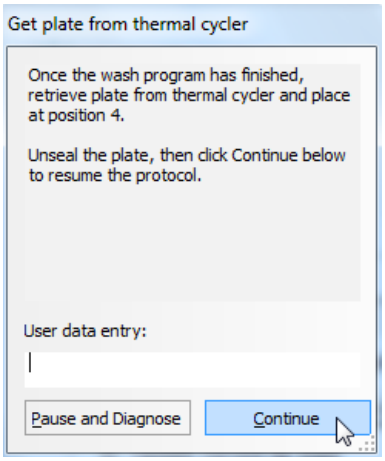

Add the volume of Ligation Master Mix indicated in Table 30 to all wells of column 2 of the Nunc DeepWell Master Mix source plate on Bravo deck position 9.

**Table 30** Addition of Ligation Master Mix to the Master Mix Source Plate for Capture_Exome.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. Be sure to add the Ligation Master Mix to Column 2 of the source plate at this step.

**10** Once the wash program in Table 29 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_Exome.pro protocol.

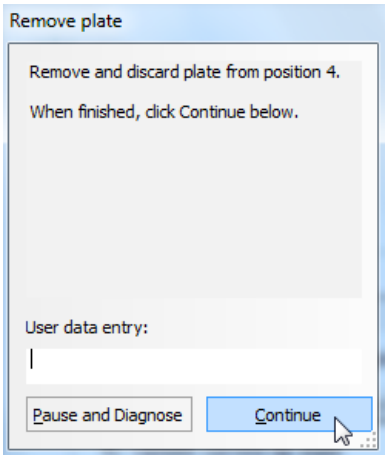



The NGS Bravo completes the liquid-handling steps for ligation of the captured target DNA.

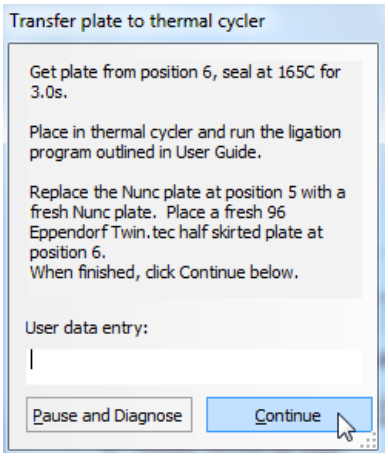
**NOTE**

To expedite thermal cycler warm-up for the subsequent ligation program on page 69, you can enter and initiate the 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 12.

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



**12** 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 31, using a heated lid.

**Table 31** Thermal cycler program for Capture_Exome.pro ligation step

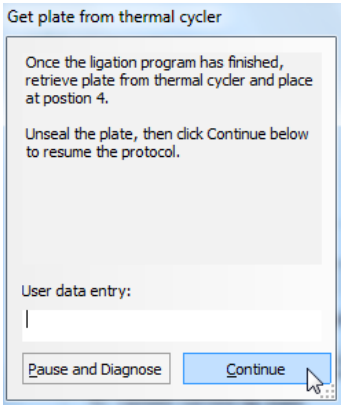
| Step | Temperature | Time |
|---|---|---|
| Step 1 | 55°C | 10 minutes |
| Step 2 | 4°C | Hold |

**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.

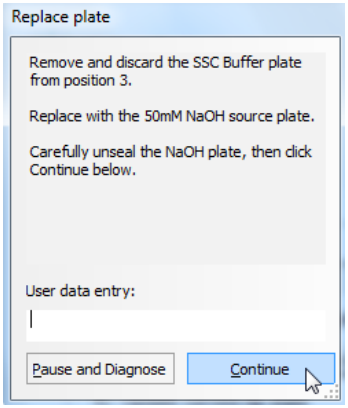
**d** Place a fresh half-skirted 96-well Eppendorf twin.tec plate at position 6 for use in the following PCR protocol steps.

**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.

13 Once the ligation program in Table 31 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_Exome.pro protocol.




14 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.

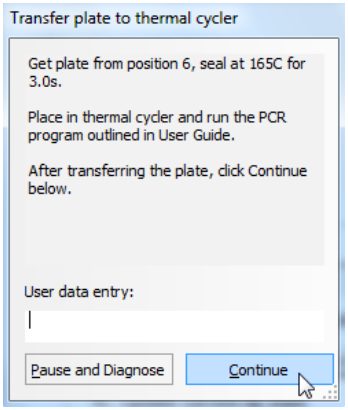



The NGS Bravo completes the liquid-handling steps for elution of the captured target DNA followed by preparation of PCR reactions for amplification.

**NOTE**

To expedite thermal cycler warm-up for the subsequent PCR program on page 72, you can enter and initiate the PCR program on the thermal cycler now, and then pause the program until you are instructed to transfer the reaction plate. Be sure to pause the thermal cycler before the initiation of the 98°C denaturation segment. Release the pause immediately after transferring the plate to the thermal cycler in step 15.

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




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

**b** Transfer the sealed plate to a thermal cycler and run the PCR program in Table 32, using a heated lid.

Consult the Certificate of Analysis (provided with HaloPlex Exome Target Enrichment System Box 1) for the PCR cycling recommendation.

**Table 32** 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 73. 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 5. 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 NucleoClean 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.

**NOTE**

After preparing the source plate, retain the beads at room temperature for use in the next step of the workflow on page 77.

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 NGS Bravo and Run the Purification_01_Exome.pro VWorks Protocol

1. On the VWorks HaloPlex form, under **Step**, select **05 Purification_01_Exome.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 33.

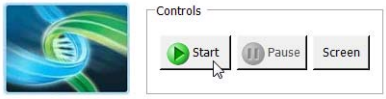
**Table 33** Initial Bravo deck configuration for Purification_01_Exome.pro

| Location | Content |
|---|---|
| 1 | Empty Axygen 96 Deep Well Plate (square wells) for waste |
| 2 | New tip box |
| 3 | —(empty)— |
| 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.

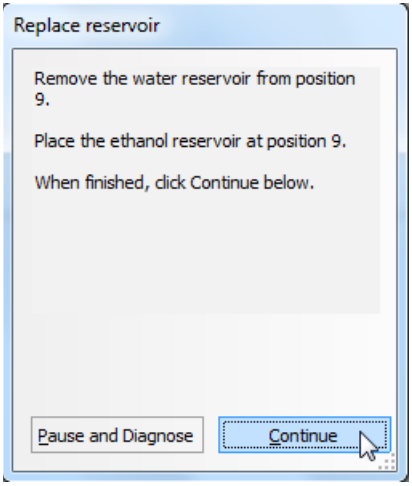
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 19 for more information on using this segment of the form during the run.

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



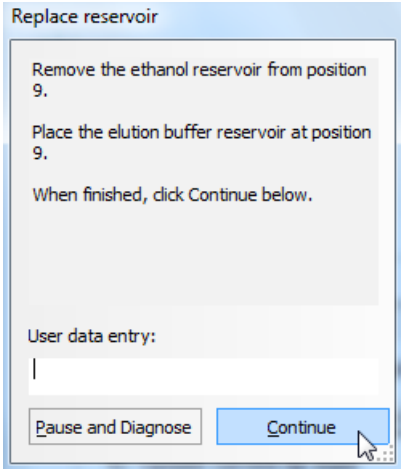
8 When prompted by VWorks as shown below, remove the water reservoir from position 9 of the Bravo deck and replace it with the 70% ethanol reservoir.

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.

When the automation protocol is finished, the DNA samples are in the bead-containing Nunc DeepWell plate at position 7. Leave the sample plate on the Bravo deck for the subsequent automation protocol, **Purification_02_Exome.pro.**

# Step 6. Remove primer dimers in second serial purification

In this step, the NGS Bravo does the liquid handling steps to remove primer dimers from the enriched DNA library using AMPure XP beads.

1. Verify that the AMPure XP beads are at room temperature. If beads are in cold storage, let beads come to room temperature for at least 30 minutes.
2. Mix the room-temperature bead suspension well so that the reagent appears homogeneous and consistent in color.
3. 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.
4. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
5. 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)].
6. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

### Load the NGS Bravo and Run the Purification_02_Exome.pro. VWorks Protocol

1. On the VWorks HaloPlex form, under **Step**, select **06 Purification_02_Exome.pro.**
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Click **Update layout and information.**

4 Load the Bravo deck according to Table 34.

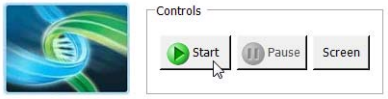
**Table 34** Initial Bravo deck configuration for Purification_02_Exome.pro.

| 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 | —(empty)— |
| 7 | Library samples for purification in Nunc DeepWell plate |
| 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.

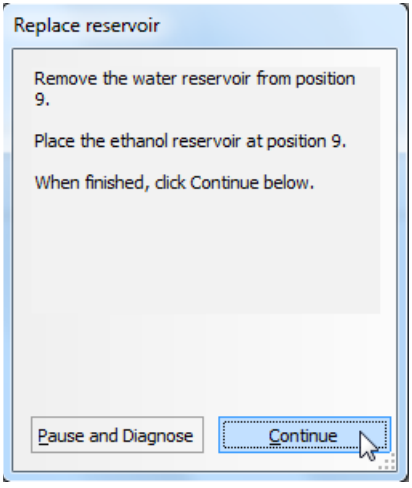
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 19 for more information on using this segment of the form during the run.

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



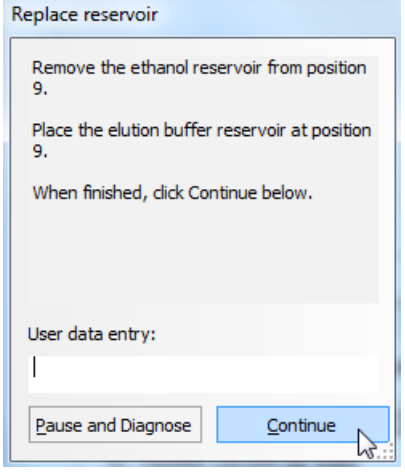
8 When prompted by VWorks as shown below, remove the water reservoir from position 9 of the Bravo deck and replace it with the 70% ethanol reservoir.

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



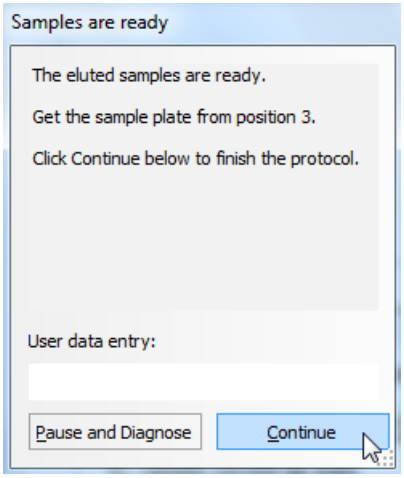

9 When prompted by VWorks as shown below, remove the 70% ethanol reservoir from position 9 of the Bravo deck and replace it with the final sample elution buffer reservoir.

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 7. Validate enrichment and quantify enriched target DNA

Prior to sample pooling and sequencing sample preparation, validate enrichment and quantify the enriched target DNA in each library sample by microfluidics analysis using the 2100 Bioanalyzer (see page 82) or the 2200 TapeStation (see page 83).

Enriched library samples may also be qualitatively analyzed using gel electrophoresis. Sample gel electrophoresis results are provided in the Reference section on page 105.

### 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 8.

**Figure 8** Content of HaloPlex-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (yellow), the sample index (green) and the library bridge PCR primers (gray).

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

### Option 1: Analysis using the 2100 Bioanalyzer System

Use a Bioanalyzer High Sensitivity DNA Assay kit and the 2100 Bioanalyzer instrument with 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer instrument and assay setup instructions.

1 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of enriched library sample for the analysis.

2 Load the prepared chip into the 2100 Bioanalyzer instrument and start the run within five minutes after preparation.

3 Analyze the electropherogram for each sample according to the analysis guidelines on page 84.

See Figure 9 for a sample Bioanalyzer 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.

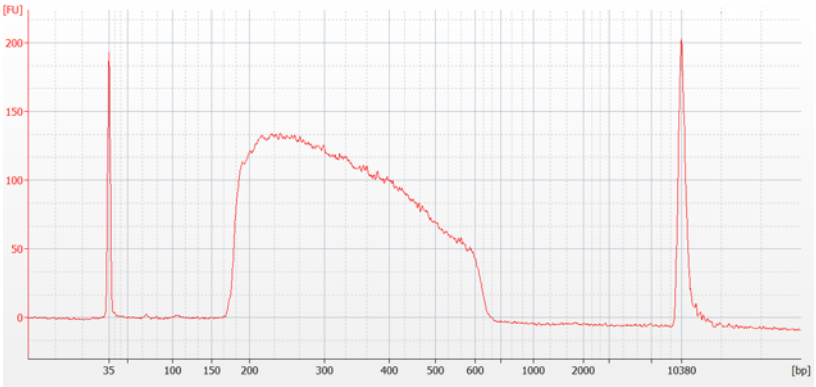



**Figure 9** Validation of HaloPlex enrichment by 2100 Bioanalyzer system analysis.

### Option 2: Analysis using the 2200 TapeStation

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the enriched library samples. For more information to do this step, see the *2200 TapeStation User Manual*.

1 Prepare the TapeStation samples as instructed in the *2200 TapeStation User Manual*. Use 2 µL of each enriched library sample diluted with 2 µL of High Sensitivity D1000 sample buffer in separate wells of a tube strip for the analysis.

**CAUTION** Make sure that you thoroughly mix the combined DNA sample and High Sensitivity D1000 sample buffer on a vortex mixer for 5 seconds for accurate results.

2 Load the sample tube strip, the High Sensitivity D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *2200 TapeStation User Manual*. Start the run.

3 Analyze the electropherogram for each sample according to the analysis guidelines on page 84.

See Figure 10 for a sample TapeStation electropherogram.

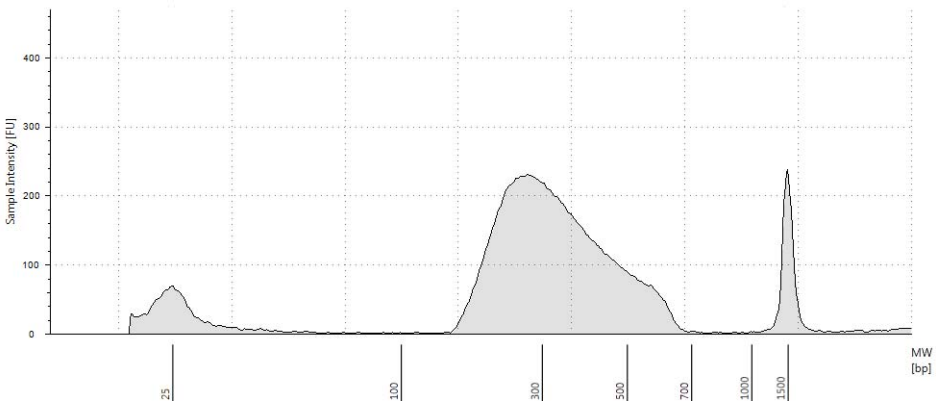



**Figure 10** Validation of HaloPlex enrichment by 2200 TapeStation analysis.

### Analysis of Electropherogram Results

- Check that the electropherogram shows a peak fragment size between approximately 225 to 525 bp.
- Determine the concentration of enriched target DNA in the sample by integration under the peak between 175 and 625 bp. Peaks at <150 bp may be observed, but should be excluded from quantitation.

# Step 8. Pool samples with different indexes for multiplexed sequencing

Use the following guidelines to design your sample pooling strategy:

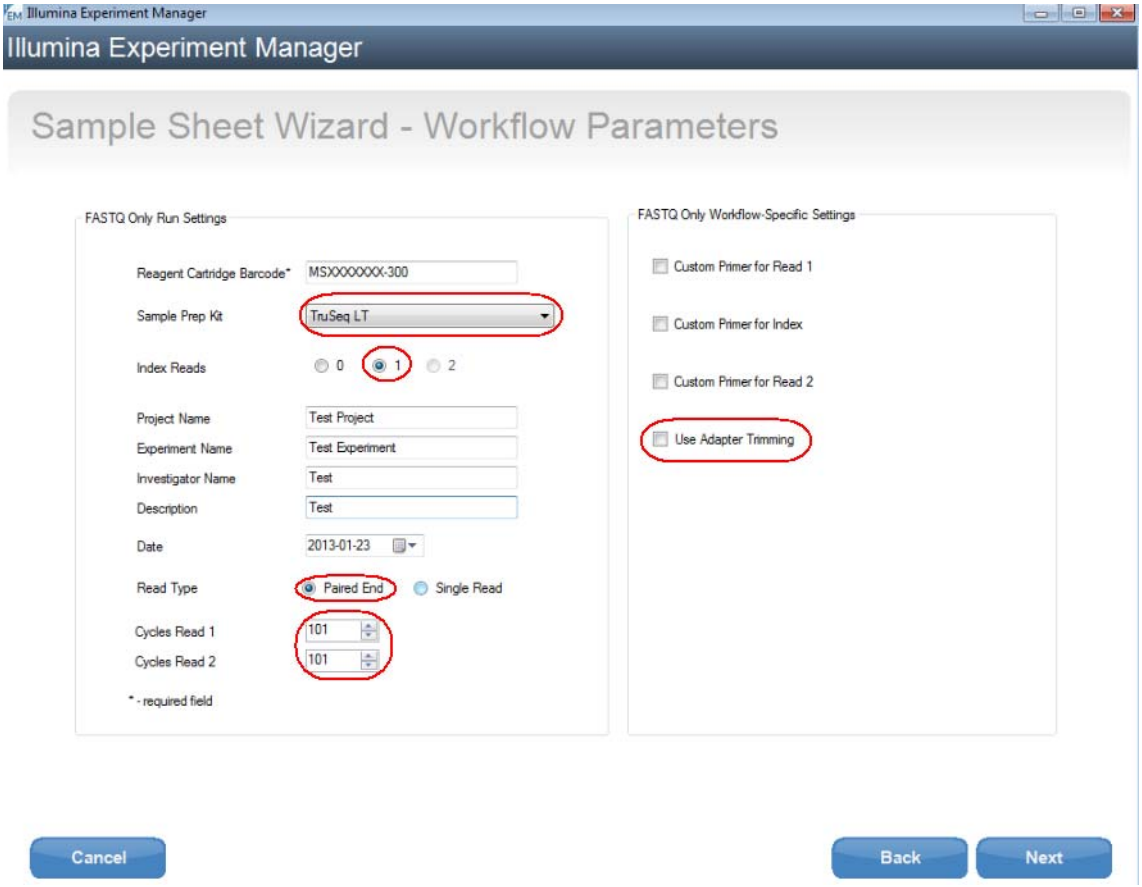
- Use the Bioanalyzer- or TapeStation-measured concentration of 175-625 bp products in each sample to pool equimolar amounts of differentially indexed samples in order to optimize the use of sequencing capacity.
- The final HaloPlex enrichment pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry on the Illumina HiSeq, MiSeq, or GAIIx platform. See additional guidelines for the MiSeq platform (below) and HiSeq platform (page 89).
- Use 100 + 100 bp read length paired-end sequencing.
- Sequencing runs must be set up to perform an 8-nt index read. For complete index sequence information, see the Reference chapter starting on page 91.
- Before aligning reads to the reference genome, trim the reads from Illumina adaptor sequences.

### MiSeq platform sequencing run setup guidelines

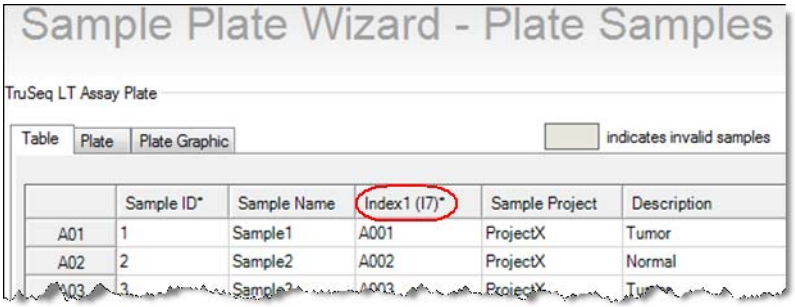
#### Setting up a custom Sample Sheet:

1 In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.
   - Under **Category**, select *Other*.
   - Under **Application**, select *FASTQ Only*.

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



3 Using the **Sample Plate Wizard**, set up a New Plate, entering the required information for each sample to be sequenced. In the **Index 1(17)** column of the **TrueSeq LT Assay Plate** table, assign each sample to any of the Illumina 17 indexes. The index will be corrected to a HaloPlex index at a later stage.




4 Finish the sample plate setup tasks and save the sample plate file.

5 Using the **Sample Sheet Wizard**, select the samples to include in the run and save the Sample Sheet file.

**Editing the Sample Sheet to Include HaloPlex indexes:**

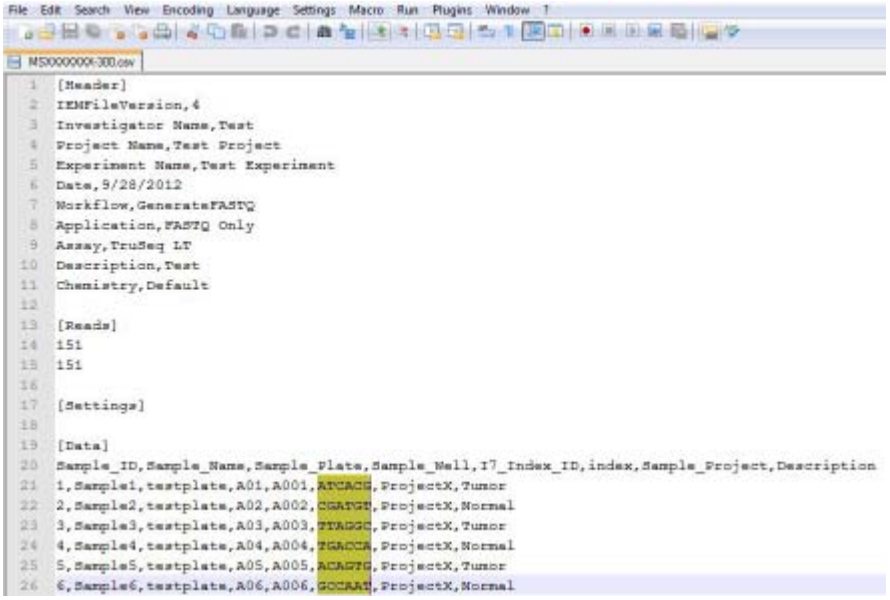
**CAUTION**

This guide includes information for kits containing two different sets of indexing primers. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Kits with indexing primers supplied in a blue plate include indexing primers A01–H12. See page 95 for index nucleotide sequence information.

Kits with indexing primers supplied in a clear plate include indexing primers 1–96. See page 99 through page 104 for index nucleotide sequence information.

1 Open the Sample Sheet file in a text editor. For each sample, select the text for the 6-nucleotide index (highlighted below), and replace with the appropriate 8-nucleotide HaloPlex index sequence.




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

### HiSeq platform sequencing run setup guidelines

Set up sequencing runs to perform an 8-nt index read using the *Cycles* settings shown in Table 35. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

**Table 35** HiSeq platform Run Configuration screen Cycle Number settings*

| Run Segment | Cycle Number |
|---|---|
| Read 1 | 100 |
| Index 1 (i7) | 9 |
| Index 2 (i5) | 0 |
| Read 2 | 100 |

* Settings apply to v3.0 SBS chemistry.

### Sequence analysis resources

Agilent's SureCall data analysis software is available to simplify the sequencing data analysis workflow after HaloPlex target enrichment. To learn more about this resource and download the SureCall software free of charge, visit www.agilent.com/genomics/surecall.

# 4 Reference



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

**CAUTION**

This chapter contains two sets of index sequence and kit content information. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

The first section covers kits with reconfigured indexing primers, typically received December, 2014 or later. The reconfigured primers A01–H12 are supplied in a blue plate. See page 92 through page 95 for details.

The second section covers kits with original indexing primer configuration, typically received before December, 2014. The original configuration includes primers 1–96, supplied in a clear plate. See page 96 through page 104 for details.

# Reference Information for Kits with Revised Index Configuration (indexing primers in blue plate)

If your kit includes indexing primers in a clear plate, instead see page 96 for kit content and indexing primer information.

## Kit Contents-Revised Configuration

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

**Table 36** HaloPlex Exome Target Enrichment System Kit Contents

| Kit Type | HaloPlex Exome, ILM, Box 1* | HaloPlex Magnetic Beads, Box 2 |
|---|---|---|
| | Store at –20°C | Store at +4°C |
| **HaloPlex Exome, ILM, 96 Reactions, Automated** | 5190-8064 | 5190-5386 |

* See Table 37 for list of included reagents.

The contents of the HaloPlex Exome Target Enrichment System Box 1 (revised index configuration) included with each kit are detailed in the table below:

**Table 37** HaloPlex Exome 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 |
| BSA 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 Primer Cassettes | 96-well plate with Indexing Primer Cassettes 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 8-well Strip | 8-well strip tube with black label |

* See Table 38 for a plate map.

**Table 38 Plate map for HaloPlex Indexing Primer Cassettes 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 (indexing primers in blue plate)

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex Indexing Primer Cassette (revised index configuration) is provided in the table below.

**Table 39 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 |

# Reference Information for Kits with Original Index Configuration (indexing primers in clear plate)

If your kit includes indexing primers in a blue plate, instead see page 92 for kit content and indexing primer information.

## Kit Contents-Original Configuration

The HaloPlex Exome Target Enrichment System (original index configuration) includes the following component kits:

**Table 40** HaloPlex Exome Kit Contents

| Kit Type | HaloPlex Exome, ILM, Box 1* | HaloPlex Magnetic Beads, Box 2 |
|---|---|---|
| | Store at –20°C | Store at +4°C |
| **HaloPlex Exome, ILM, 96 Reactions, Automated** | 5190-6291 | 5190-5386 |

* See Table 41 for list of included reagents.

The contents of the HaloPlex Exome Target Enrichment System Box 1 (original index configuration) included with each kit are detailed in the table below:

**Table 41** HaloPlex Exome 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 |
| BSA 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 Primer Cassettes | 96-well plate with Indexing Primer Cassettes 1-96 (clear plate)* |
| Enzyme Strip 1 | 8-well strip tube with green label |
| Enzyme Strip 2 | 8-well strip tube with red label |
| HaloPlex Probe 8-well Strip | 8-well strip tube with black label |

* See Table 42 for a plate map.

**Table 42 Plate map for HaloPlex Indexing Primer Cassettes 1-96 provided in clear plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Index 1 | Index 9 | Index 17 | Index 25 | Index 33 | Index 41 | Index 49 | Index 57 | Index 65 | Index 73 | Index 81 | Index 89 |
| **B** | Index 2 | Index 10 | Index 18 | Index 26 | Index 34 | Index 42 | Index 50 | Index 58 | Index 66 | Index 74 | Index 82 | Index 90 |
| **C** | Index 3 | Index 11 | Index 19 | Index 27 | Index 35 | Index 43 | Index 51 | Index 59 | Index 67 | Index 75 | Index 83 | Index 91 |
| **D** | Index 4 | Index 12 | Index 20 | Index 28 | Index 36 | Index 44 | Index 52 | Index 60 | Index 68 | Index 76 | Index 84 | Index 92 |
| **E** | Index 5 | Index 13 | Index 21 | Index 29 | Index 37 | Index 45 | Index 53 | Index 61 | Index 69 | Index 77 | Index 85 | Index 93 |
| **F** | Index 6 | Index 14 | Index 22 | Index 30 | Index 38 | Index 46 | Index 54 | Index 62 | Index 70 | Index 78 | Index 86 | Index 94 |
| **G** | Index 7 | Index 15 | Index 23 | Index 31 | Index 39 | Index 47 | Index 55 | Index 63 | Index 71 | Index 79 | Index 87 | Index 95 |
| **H** | Index 8 | Index 16 | Index 24 | Index 32 | Index 40 | Index 48 | Index 56 | Index 64 | Index 72 | Index 80 | Index 88 | Index 96 |

# Nucleotide Sequences of HaloPlex Indexes (indexing primers in clear plate)

The nucleotide sequence of the 8-nucleotide index portion of each HaloPlex Indexing Primer Cassette (original index configuration) is provided in the Table 43 to Table 48 below.

**Table 43** HaloPlex Indexes 1-16

| Index Number | Sequence |
|---|---|
| 1 | AACGTGAT |
| 2 | AAACATCG |
| 3 | ATGCCTAA |
| 4 | AGTGGTCA |
| 5 | ACCACTGT |
| 6 | ACATTGGC |
| 7 | CAGATCTG |
| 8 | CATCAAGT |
| 9 | CGCTGATC |
| 10 | ACAAGCTA |
| 11 | CTGTAGCC |
| 12 | AGTACAAG |
| 13 | AACAACCA |
| 14 | AACCGAGA |
| 15 | AACGCTTA |
| 16 | AAGACGGA |

**Table 44** HaloPlex Indexes 17-32

| Index Number | Sequence |
|---|---|
| 17 | AAGGTACA |
| 18 | ACACAGAA |
| 19 | ACAGCAGA |
| 20 | ACCTCCAA |
| 21 | ACGCTCGA |
| 22 | ACGTATCA |
| 23 | ACTATGCA |
| 24 | AGAGTCAA |
| 25 | AGATCGCA |
| 26 | AGCAGGAA |
| 27 | AGTCACTA |
| 28 | ATCCTGTA |
| 29 | ATTGAGGA |
| 30 | CAACCACA |
| 31 | CAAGACTA |
| 32 | CAATGGAA |

**Table 45** HaloPlex Indexes 33-48

| Index Number | Sequence |
|---|---|
| 33 | CACTTCGA |
| 34 | CAGCGTTA |
| 35 | CATACCAA |
| 36 | CCAGTTCA |
| 37 | CCGAAGTA |
| 38 | CCGTGAGA |
| 39 | CCTCCTGA |
| 40 | CGAACTTA |
| 41 | CGACTGGA |
| 42 | CGCATACA |
| 43 | CTCAATGA |
| 44 | CTGAGCCA |
| 45 | CTGGCATA |
| 46 | GAATCTGA |
| 47 | GACTAGTA |
| 48 | GAGCTGAA |

**Table 46** HaloPlex Indexes 49-64

| Index Number | Sequence |
|---|---|
| 49 | GATAGACA |
| 50 | GCCACATA |
| 51 | GCGAGTAA |
| 52 | GCTAACGA |
| 53 | GCTCGGTA |
| 54 | GGAGAACA |
| 55 | GGTGCGAA |
| 56 | GTACGCAA |
| 57 | GTCGTAGA |
| 58 | GTCTGTCA |
| 59 | GTGTTCTA |
| 60 | TAGGATGA |
| 61 | TATCAGCA |
| 62 | TCCGTCTA |
| 63 | TCTTCACA |
| 64 | TGAAGAGA |

**Table 47** HaloPlex Indexes 65-80

| Index Number | Sequence |
|---|---|
| 65 | TGGAACAA |
| 66 | TGGCTTCA |
| 67 | TGGTGGTA |
| 68 | TTCACGCA |
| 69 | AACTCACC |
| 70 | AAGAGATC |
| 71 | AAGGACAC |
| 72 | AATCCGTC |
| 73 | AATGTTGC |
| 74 | ACACGACC |
| 75 | ACAGATTC |
| 76 | AGATGTAC |
| 77 | AGCACCTC |
| 78 | AGCCATGC |
| 79 | AGGCTAAC |
| 80 | ATAGCGAC |

**Table 48** HaloPlex Indexes 81-96

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

# Qualitative analysis of enrichment by gel electrophoresis

Enrichment products may be qualitatively analyzed by gel electrophoresis. Analyze 5 µL of each enriched library sample (enriched ECD sample or experimental enriched libraries) by electrophoresis on a Novex 6% polyacrylamide TBE pre-cast gel. See page 42 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 11 for a sample gel analysis image.

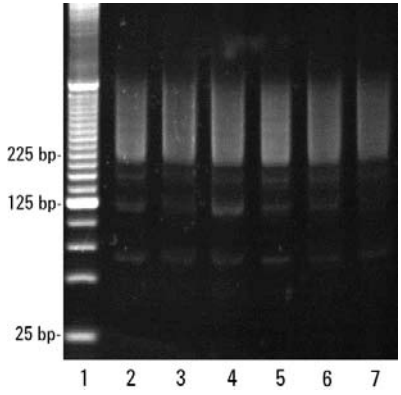



**Figure 11** Validation of HaloPlex enrichment process by gel electrophoresis. Lane 1: 25-bp DNA ladder, Lanes 2-7: enriched library samples.

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## In This Book

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



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