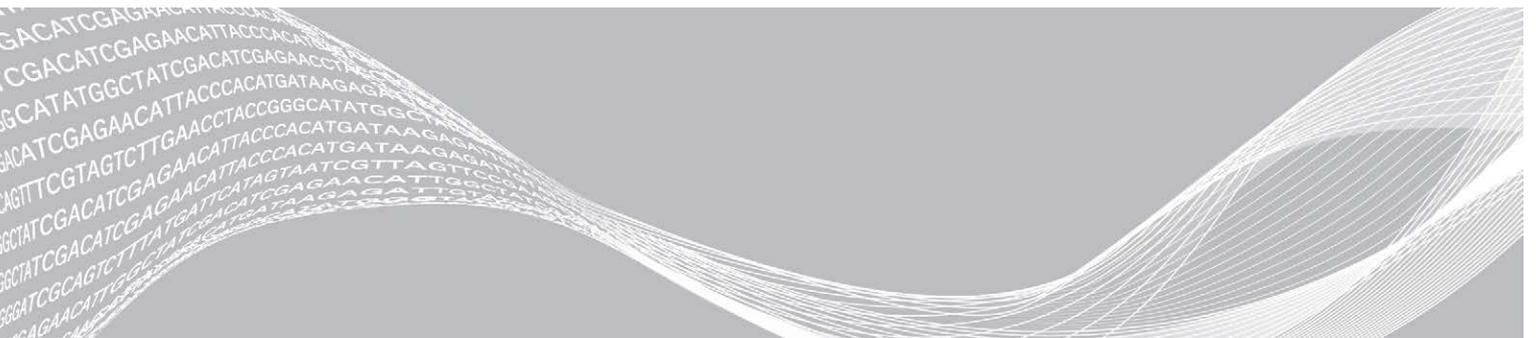


HiSeq 4000

System Guide



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Revision History

Document	Date	Description of Change
Material # 20015630 Document # 15066496 v06	May 2019	Updated index read information. Re-added SBS Kits for Dual-Indexed Sequencing information which was previously moved to the support site. Corrected HP14 reagent information back to HP12. Minor text edits.
Material # 20015630 Document # 15066496 v05	March 2018	Added information about the Illumina Proactive monitoring service in the View and Send Instrument Data section. Updated reagent information to replace HP12 with HP14.
Material # 20015630 Document # 15066496 v04	January 2017	Updated the maintenance wash procedure. Removed Sigma-Aldrich catalog # SRE0076 for SeqClin Wash Solution. Updated software name to HiSeq Control Software HD v3.4.
Material # 20013047 Document # 15066496 v03	September 2016	Added Custom Protocol Selector to Additional Resources. Added Sigma-Aldrich catalog # SRE0076 for SeqClin Wash Solution. Noted approximate frequency for renewing wash bottles and tubes. Updated instructions for starting the instrument: <ul style="list-style-type: none"> • Wait for the system to load before logging on to the operating system, not after. • Increased the duration for instrument devices to be configured and DoNotEject to initialize from 1 minute to 3 minutes. • Noted that hard drives must be empty for proper operation. Updated quick format instructions to include the scratch (S:) drive. Corrected instructions for accessing the log file. Corrected descriptions of BaseSpace Server options.

Document	Date	Description of Change
Material # 20007157 Document # 15066496 v02	May 2016	<p>Updated software descriptions for HiSeq Control Software v3.3.76:</p> <ul style="list-style-type: none"> • Added instructions for BaseSpace Enterprise subscribers to configure a domain. • Updated the instructions for staggering runs to include use of the Pause button. <p>Renamed BaseSpace to BaseSpace Sequence Hub and BaseSpace Onsite to BaseSpace Onsite Sequence Hub.</p> <p>Added the following information:</p> <ul style="list-style-type: none"> • Catalog numbers for cluster kits. • The <i>cBot 2 System Guide (document # 15065681)</i> as a reference for clustering. • The folder structure for sequencing output files and the run folder name and path. • Troubleshooting loss of registration in Read 1. • Recommendation for annual preventive maintenance service. <p>Removed the following items:</p> <ul style="list-style-type: none"> • Self-standing conical tubes and pipette tips from user-supplied consumables. • The default user name and password required to log on to the operating system. Illumina recommends using site-specific credentials. • The catalog number for this guide. <p>Corrected the following items:</p> <ul style="list-style-type: none"> • Instructions for preparing and loading indexing and paired-end reagents for a single-read run. HRM is not required for a single-index single-read run. • The name of the Real-Time Analysis software used on the instrument from RTA v2 to RTA2.
Material # 20000063 Document # 15066496 v01	August 2015	<p>Updated software descriptions to HiSeq Control Software v3.3.52, which adds the following features:</p> <ul style="list-style-type: none"> • The option to sequence a single-read flow cell. • The option to connect to BaseSpace Onsite. • Sensor indicators showing the data transfer status of Run Copy Service and BaseSpace. <p>Renamed the PE Reagent Kit ID field on the Reagents screen to Cluster Kit ID field.</p> <p>Updated instructions for staggering runs on flow cell A and flow cell B. Added option to specify a custom number of SBS cycles from the Reagents screen, and updated cycles remaining defaults.</p> <p>Added the following information:</p> <ul style="list-style-type: none"> • Instructions for preparing SBS, paired-end, and indexing reagents. • Instruction to wipe the flow cell holder with water before wiping with alcohol. • Note to dispose of maintenance wash solution according to local government standards. <p>Corrected duration and expected volumes for the maintenance wash.</p>
Part #15066496 Rev. A	February 2015	Initial release.

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Chapter 1 Overview

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Introduction

The HiSeq[®] 4000 system combines innovative engineering with proven performance to deliver maximum throughput and lowest cost for production-scale genomics.

Features

- ▶ **Dual-surface imaging**—The HiSeq 4000 uses a two-camera, four-sensor epifluorescence system with cutting-edge scanning technology to enable dual surface imaging.
- ▶ **Patterned flow cell**—A patterned flow cell allows the generation of sequencing clusters in an ordered arrangement, which increases output reads and data.
- ▶ **Dual flow cells**—The HiSeq 4000 is a dual flow cell system, which allows sequencing of a single flow cell or 2 flow cells with different read lengths simultaneously.
- ▶ **High-capacity reagent chiller**—The reagent compartment is a high-capacity chiller that holds enough reagents for the entire sequencing run.
- ▶ **Integrated fluidics for paired-end runs**—Integrated paired-end fluidics provide reagents from the reagent compartment to the flow cell for Read 2 resynthesis and for indexed sequencing.
- ▶ **Interface control options**—The instrument software interface provides options for setting up a run and operating the instrument. Use the touch screen monitor or the integrated keyboard to provide input.
- ▶ **Real-time base calling**—The instrument software extracts intensities from images and performs quality-scored base calling on the instrument computer. This method allows monitoring of quality metrics during the run and saves time during subsequent data analysis.
Downstream analysis of sequencing data can be performed with Illumina[®] analysis software or third-party software on a custom infrastructure.
- ▶ **BaseSpace[®] Sequence Hub integration**—The sequencing workflow is integrated with BaseSpace Sequence Hub, the Illumina genomics computing environment for data analysis, storage, and collaboration. As the run progresses, output files are streamed in real time to BaseSpace Sequence Hub or BaseSpace Onsite Sequence Hub.

Additional Resources

The following documentation is available for download from the Illumina website. Always check support pages for the latest versions.

Resource	Description
Custom Protocol Selector	A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
HiSeq 4000 and HiSeq 3000 Systems Site Prep Guide (document # 15066492)	Provides specifications for laboratory space, electrical requirements, and environmental considerations.

Resource	Description
<i>HiSeq 4000 and HiSeq 3000 Systems Safety and Compliance Guide (document # 15066491)</i>	Provides information about instrument labeling, compliance certifications, and safety considerations.

Visit the HiSeq 4000 support page on the Illumina website for access to documentation, software downloads, online training, and frequently asked questions.

Instrument Components

The HiSeq 4000 system comprises the instrument, monitor, instrument control computer, and accessories, such as a keyboard, mouse, and barcode scanner. The instrument includes four main compartments: the optics module, flow cell compartment, fluidics compartment, and reagents compartment. An illuminated status bar indicates operating status.

Figure 1 External Components



- A **Optics module**—Contains optical components that enable dual surface imaging of the flow cell, imaging A, C, G, and T at the same time using epifluorescence. The excitation laser beam passes through the objective and the fluorescence is simultaneously collected through the same objective.
- B **Flow cell compartment**—Contains the vacuum-controlled flow cell stage, which holds the flow cell in place during sequencing runs.
- C **Fluidics compartment**—Contains fluidics pumps that deliver reagents to the flow cell, and then to the waste container.
- D **Status bar**—Uses three colors to indicate instrument status. Blue indicates that the instrument is running, orange indicates that the instrument needs attention, and green indicates that the instrument is ready to begin the next run.
- E **Reagent compartment**—Contains reagent racks that hold reagents for sequencing runs and wash solution for instrument washes.

Flow Cell Compartment

The flow cell compartment houses the flow cell stage, the thermal stations, the vacuum system, and the fluidics connections to each flow cell.

Figure 2 Flow Cell Stage With Two Flow Cells

- A Flow cell A
- B Flow cell B
- C Flow cell lever A
- D Flow cell lever B

Flow cell A is on the left, and flow cell B is on the right. Each flow cell is seated on the flow cell stage, which moves in and out of the optics module as directed by the control software. The flow cell stage must be in the forward-most position to open the flow cell compartment door and load or remove a flow cell.

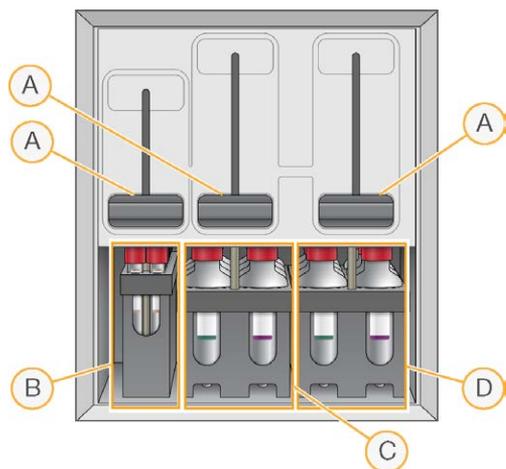
The flow cell is positioned on the flow cell holder with the inlet and outlet ports facing down. A vacuum beneath the flow cell holder holds the flow cell in place. The illuminated flow cell lever in front of each flow cell holder controls the vacuum. The flow cell lever turns green when the vacuum seal is secure.

Reagent Compartment

The reagent compartment is a high-capacity reagent chiller that holds three reagent racks: two for SBS reagents and 1 for indexing and paired-end reagents. Sipper handles lower the sippers into the reagent bottles.

- ▶ **SBS reagent racks**—Hold 250 ml conical bottles. The reagent rack for flow cell A is in the center position, and the rack for flow cell B is in the far right position. Each reagent rack has numbered positions that correspond to connections on an internal reagent selector valve.
- ▶ **Indexing and paired-end reagent rack**—Located in the left position. It has two rows of numbered positions that hold 15 ml conical tubes containing paired-end reagents and indexing reagents. The left row is for flow cell A, and the right row is for flow cell B.
- ▶ **Reagent chiller**—The reagent chiller houses the reagent racks and maintains an internal temperature of 2°C to 8°C.

Figure 3 Reagent Compartment



- A Sipper handles
- B Reagent rack for indexing and paired-end reagents
- C Reagent rack for SBS reagents for flow cell A
- D Reagent rack for SBS reagents for flow cell B

HiSeq 4000 Software

Three software applications are installed on the instrument computer:

- ▶ **HiSeq 4000 control software**—The HiSeq Control Software HD v3.4 interface guides you through the steps to set up a sequencing run. During the run, the control software operates instrument hardware, controls fluidics, sets temperatures, and provides a visual summary of quality statistics.
- ▶ **Real-Time Analysis software**—Integrated with the control software, Real-Time Analysis performs base calling and assigns a quality score to each base for each cycle. For more information, see *Real-Time Analysis* on page 36.
- ▶ **Sequencing Analysis Viewer software**—Sequencing Analysis Viewer (SAV) provides detailed quality statistics.

Status Icons

A status icon located in the upper-right corner of each screen shows changes in conditions, errors, or warnings during run setup and during the run.

Status Icon	Status Name	Description
	Status okay	No change. System is normal.
	Information	Information only. No action is required
	Attention	Information that might require attention.

Status Icon	Status Name	Description
	Warning	Warnings do not stop a run, but might require action before proceeding.
	Error	Errors usually stop a run and generally require action before proceeding with the run.

When a change in condition occurs, the associated icon blinks to alert you.

- ▶ Select the icon to open the status window and view a description of the condition.
- ▶ Select **Acknowledge** to accept the message and **Close** to close the dialog box.

Activity and Sensor Indicators

The Welcome screen contains a series of icons in the lower-right corner of the screen. The icons indicate instrument activity and status of specific components based on instrument sensors.

Figure 4 Activity Indicators



From left to right, activity indicators represent the X, Y, and Z motors, electronics functionality, the camera, the fluidics system, and processing functions.

Figure 5 Sensor Indicators



From left to right, sensor indicators represent flow cell A temperature, reagent chiller temperature, data transfer status, BaseSpace Hub cloud status, and flow cell B temperature.

Data Transfer Status

The HiSeq software suite includes Run Copy Service, which manages data transfer to the output folder. A BaseSpace option sends instrument health and sequencing data to BaseSpace Sequence Hub or BaseSpace Onsite Sequence Hub.

Two of the sensor indicators on the software interface show the transfer status of Run Copy Service and BaseSpace Sequence Hub.

Run Copy Service

The transfer status of Run Copy Service affects whether you can start a new run or safely format the output drive.

Status Icon	Description
	Data are transferring. Do not format the output drive until transfer is complete.
	Data are transferring but the network connection is slow. You can set up a sequencing run and format the output drive when transfer is complete.
	Run Copy Service is off.
	Run Copy Service is on but not transferring data.

BaseSpace Sequence Hub

A BaseSpace sensor indicator shows the status of BaseSpace Sequence Hub. A blue cloud indicates an active connection. A gray cloud indicates that the software cannot connect. The following table provides additional details about each status icon.

Status Icon	Description
	Not connected to BaseSpace Sequence Hub.
	Connected to BaseSpace Sequence Hub but not transferring data.
	Connected to BaseSpace Sequence Hub and transferring data for four runs or less.
	Connected to BaseSpace Sequence Hub and transferring data for five or more runs. While this icon is displayed, the control software does not allow any new runs to connect to BaseSpace Sequence Hub.
	Disconnected from BaseSpace Sequence Hub with data queued for transfer.

Sequencing Consumables Overview

Performing a run on the HiSeq 4000 requires one HiSeq 3000/4000 SBS Kit and one cluster kit. Cluster kits are available in paired-end (PE) and single-read (SR) versions.

Kit Name	Catalog #
HiSeq 3000/4000 SBS Kit (300 cycles)	FC-410-1003
HiSeq 3000/4000 SBS Kit (150 cycles)	FC-410-1002
HiSeq 3000/4000 SBS Kit (50 cycles)	FC-410-1001
HiSeq 3000/4000 PE Cluster Kit	PE-410-1001
HiSeq 3000/4000 SR Cluster Kit	GD-410-1001

SBS kits include sequencing reagents used on the HiSeq, with sufficient reagents for sequencing one flow cell. Sequencing reagents are provided in 250 ml bottles that load directly onto reagent racks. The reagent labels are color-coded to reduce loading errors.

Cluster kits contain clustering reagents used on the cBot and indexing and paired-end reagents used on the HiSeq 4000. Each cluster kit includes an accessory kit that provides flow cell gaskets, funnel caps for SBS reagent bottles, and a flow cell storage tube.

SBS Kits for Dual-Indexed Sequencing

HiSeq SBS kits contain a sufficient volume of reagents for performing dual-indexed runs:

- ▶ One 300-cycle SBS kit provides reagents for up to 325 cycles of sequencing.
- ▶ One 150-cycle SBS kit provides reagents for up to 174 cycles of sequencing.
- ▶ One 50-cycle SBS kit provides reagents for up to 74 cycles of sequencing.
- ▶ For runs greater than the maximum number of cycles in a kit and to maximize reagent use for other read lengths, use multiple 50-cycle kits.

The following table lists the number of cycles performed for dual-indexed paired-end runs of various lengths. Run cycles comprise several sequencing cycles based on run length and 2 index reads of 8 cycles each. For dual-indexed single-read runs, 7 chemistry-only cycles are calculated in the total number of cycles.

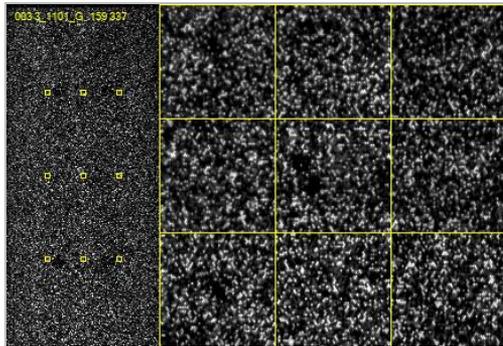
Run Length	Run Length	Run Cycles	Total Cycles
Dual-Indexed Paired End	2 x 150	151+8+8+151	318
	2 x 100	101+8+8+101	218
	2 x 75	76+8+8+76	168
	2 x 50	51+8+8+51	118
	2 x 35	36+8+8+36	88
Dual-Indexed Single-Read	1 x 150	151+8+(7)+8	174
	1 x 100	101+8+(7)+8	124
	1 x 75	76+8+(7)+8	99
	1 x 50	51+8+(7)+8	74
	1 x 35	36+8+(7)+8	59

Patterned Flow Cell

The HiSeq 4000 uses a patterned flow cell with billions of ordered nanowells that are manufactured into the glass of the flow cell. The ordered arrangement increases the number of output reads and amount of sequencing data generated.

The patterned flow cell is provided in the HiSeq 3000/4000 Cluster Kit.

Figure 6 Example of Clusters on a Patterned Flow Cell



Chapter 2 Getting Started

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Start the HiSeq 4000

- 1 Start the instrument control computer.
- 2 Wait for the system to load, and then log on to the operating system. If necessary, consult your facility administrator for the user name and password.
- 3 Locate the power switch on the left side of the instrument and switch it to the ON position.
- 4 Wait at least three minutes for the instrument devices to be configured and for the instrument drive called DoNotEject to initialize.
- 5 Close the window that opens when DoNotEject is initialized. If the window does not open, use MyComputer to check for the DoNotEject drive.



NOTE

Never eject the DoNotEject flash drive located inside the instrument chassis, or modify the files on it. This drive contains hardware configuration files and initializes whenever the instrument is turned on.

- 6 To ensure adequate disk space, archive previous run data on the instrument computer to a network location. Perform a quick reformat of the O:\ and S:\ drives to clear any remaining data. The hard drives must be empty for proper software operation.
- 7 Open HCS using the shortcut icon on the desktop. When the software has initialized, the Welcome screen opens and the Initialized icon appears in the bottom-right corner of the screen.

Instrument and Control Computer Best Practices

- ▶ Do not turn on the computer while the instrument is running. Always turn on the computer before turning on the instrument.
- ▶ Do not turn off the instrument while the instrument control software is running.
- ▶ Wait 1 minute after turning off the instrument before turning it on again.
- ▶ Connect the USB cables for the instrument, the monitor, and the keyboard to the back of the computer before turning on the computer.
- ▶ Connect the barcode scanner and mouse to the USB ports on the front of the computer.

Customize System Settings

The control software includes customizable system settings for run folders, LIMS preferences, and domains. The Menu Options window has settings to define the run ID template, default folder locations, whether to send instrument health data, LIMS authentication, and BaseSpace Enterprise domains.

To customize your view of the interface, select **Menu | View**. You can choose to view the interface in full screen or in a window, or to minimize.

Define Run Folder Settings

- 1 From the Welcome screen, select **Menu | Tools | Options** to open the Menu Options window.
- 2 To customize the naming convention for run folder names, modify the settings in the **Run ID Template** field. Select **Reset** to clear the field.
- 3 To set default output locations, enter a location for each of the following folders:
 - ▶ **Default Output Folder**—The default output folder for runs on flow cell A.
 - ▶ **Default Output Folder2**—The default output folder for runs on flow cell B.



NOTE

Illumina recommends a network location for the output folders. However, if the location differs from the HiSeq Temp folder, you can specify a location on the O:\ drive. Do not use the S:\ drive or the C:\ drive. The S:\ drive is reserved for instrument operations and the C:\ drive is too small.

- 4 To set a location for LIMS sample forms, enter the location in the **Run Setup Folder** field.
- 5 Select **OK** to save your work and close the Menu Options window. Select **Cancel** to close without saving.

Set LIMS Preferences

- 1 From the Welcome screen, select **Menu | Tools | Options** to open the Menu Options window.
- 2 Enter the following LIMS settings:
 - ▶ **LIMS Server**—The server name for interactions with supported Illumina LIMS.
 - ▶ **LIMS User Name**—The user name used when authenticating to Illumina LIMS.
 - ▶ **LIMS Password**—The password used when authenticating to Illumina LIMS.
- 3 Select **OK** to save your work and close the Menu Options window. Select **Cancel** to close without saving.

Configure a Domain

If you are a BaseSpace Enterprise subscriber, use the following instructions to configure your domain.

- 1 From the Welcome screen, select **Menu | Tools | Options** to open the Options window.
- 2 Select a BaseSpace Server option:
 - ▶ **Cloud**—Connect to your BaseSpace Sequence Hub domain.
 - ▶ **Onsite**—Connect to your BaseSpace Onsite Sequence Hub domain.
- 3 Enter the domain for the selected server.
- 4 Select **OK** to save your work and close the Options window. Select **Cancel** to close without saving.

View and Send Instrument Data

The Menu button on the Welcome screen and the Menu Options window provide options for viewing and sending instrument data.

- ▶ To view information about instrument hardware, software versions, and technical support contact information, select **Menu | About**.
- ▶ Select **Menu | Tools | Options**, and then select **Send instrument health data to Illumina to help Illumina improve its products** to enable the Illumina Proactive monitoring service. The name of the setting in the software interface might be different from the name in this guide, depending on the version of HCS in use.

With this setting turned on, instrument performance data are sent to Illumina. This data helps Illumina troubleshoot more easily and detect potential failures, enabling proactive maintenance and maximizing instrument uptime. For more information on the benefits of this service, see *Illumina Proactive Technical Note (document # 1000000052503)*.

This service:

- ▶ Does not send sequencing data.
- ▶ Requires that the instrument be connected to a network with internet access.
- ▶ Is turned on by default. To opt out of this service, disable the **Send instrument health data to Illumina to help Illumina improve its products** setting.



NOTE

This setting is turned back on after a software upgrade. If you do not want to send instrument performance data to Illumina, disable this service after every software upgrade.

User-Supplied Consumables

Consumable	Supplier	Purpose
Alcohol wipes, 70% Isopropyl or Ethanol, 70%	VWR, catalog # 95041-714 General lab supplier	Cleaning the flow cell and flow cell stage.
Carboy, at least 6 liters	General lab supplier	Preparing maintenance wash solution.
Centrifuge tubes, 250 ml	Corning, catalog # 430776	SBS reagent racks, positions containing PW1. Instrument wash.
Conical tubes, 15 ml	Corning, catalog # 430052	PE reagent racks, positions containing PW1. Instrument wash. Collecting and measuring waste volumes.
Disposable gloves, powder-free	General lab supplier	General use.
Lab tissue, low-lint	VWR, catalog # 21905-026	Cleaning the flow cell holder.
Lens paper, 4 x 6 in	VWR, catalog # 52846-001	Cleaning the flow cell.
ProClin 300, 50 ml	Sigma-Aldrich, catalog # 48912-U	Maintenance wash.
Tween 20, viscous liquid, 100 ml	Sigma-Aldrich, catalog # P7949	Maintenance wash.
Tweezers, square plastic tip	McMaster-Carr, catalog # 7003A22	Removing the flow cell gaskets.
Water, laboratory-grade, 18 MΩ	Millipore	SBS and PE reagent racks, positions containing PW1. Instrument wash.

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Introduction

Before setting up the run, prepare all reagents for sequencing: SBS reagents, indexing reagents, and paired-end reagents. All reagents are loaded when prompted by the software during run setup. Returning to the instrument during the run to reload is not necessary.

Sequencing reagents can be prepared during cluster generation.

Prepare SBS Reagents

SBS reagents are loaded onto the instrument at the beginning of the run. Use the following instructions to thaw and inspect HCM, HIM, and HSM.

Thaw SBS Reagents

- 1 Remove HCM, HIM, and HSM from -25°C to -15°C storage.
- 2 Thaw at 2°C to 8°C for about 16 hours.
Alternatively, thaw HIM and HSM in a room temperature deionized water bath for about 90 minutes. Thaw HCM in a *separate* water bath.



NOTE

Always replace your gloves after handling HCM.

- 3 Invert each bottle to mix.
- 4 Inspect HSM to make sure that no swirling patterns are visible.
- 5 Set aside HIM and HSM on ice.
- 6 Set aside HCM aside on ice *separately* to prevent cross-contamination.

Prepare Indexing and Paired-End Reagents

Indexing and paired-end reagents are loaded onto the instrument at the beginning of the run. They are used during indexing reads and the Read 2 resynthesis step of a sequencing run.

Follow the instructions to prepare indexing and paired-end reagents only if you are sequencing a paired-end flow cell or sequencing indexed libraries on a single-read flow cell.



WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Thaw and Prepare Indexing and Paired-End Reagents

- 1 Remove the following reagents from -25°C to -15°C storage:

- ▶ For a run on a paired-end flow cell—HAM, HDR, HLM2, HP11, HP14, HPM, and HRM. For non-indexed libraries, HP14 is not required.
 - ▶ For a run on a single-read flow cell:
 - ▶ Dual-indexed libraries—HDR, HP12, and HRM.
 - ▶ Single-indexed libraries—HDR and HP12.
- 2 Thaw in a beaker filled with room temperature deionized water for about 20 minutes.
 - 3 Invert each tube to mix.
 - 4 Centrifuge at 1000 rpm for 1 minute.
 - 5 Set aside HAM, HLM2, and HRM on ice.
 - 6 Set aside HDR, HP11, HP12, HP14, and HPM at room temperature.

Chapter 4 Sequencing

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Introduction

To perform a run on the HiSeq 4000, prepare all reagents and then follow the software prompts to set up the run. Run setup steps include entering run parameters, loading and priming reagents, loading the flow cell, and performing a fluidics check.

Run setup steps are organized in three tabs: Run Configuration, Pre-Run Setup, and Initiate Run.

- ▶ Run configuration screens contain drop-down lists, checkboxes, or text fields for run parameters. Use the hand-held barcode scanner to scan the flow cell or reagent kit ID, or enter the ID using the touch screen keyboard. The keyboard icon is located to the right of the text fields.
- ▶ Select **Next** to move to the next screen, or select **Back** to return to the previous screen.
- ▶ At any time during the run setup steps, select **Cancel** to exit run setup and return to the Welcome screen.

Visit [the HiSeq 4000 specifications page](#) on the Illumina website for information about run duration and other performance specifications.

Staggering Runs

You can start a new run on either flow cell A or flow cell B when a run on the adjacent flow cell is in progress. For more information, see *Stagger Runs on Flow Cell A and Flow Cell B* on page 34.

Enter Run Parameters

Begin run setup by entering run parameters from a series of screens on the Run Configuration tab. The software guides you through each screen to specify BaseSpace Sequence Hub connectivity, enter consumable IDs, select indexing options, and record other parameters.

Storage Screen

- 1 From the Welcome screen, select **Sequence** to open the Storage screen.
- 2 **[Optional]** Connect to BaseSpace Sequence Hub or BaseSpace Onsite Sequence Hub as follows.
 - a Select **Connect to BaseSpace**.
 - b Select **BaseSpace** or **BaseSpace Onsite**.
 - c If you selected BaseSpace, select from the following options:
 - ▶ **Storage and Analysis**—Sends run data to BaseSpace Sequence Hub for remote monitoring and data analysis. A sample sheet is required with this option.
 - ▶ **Run Monitoring Only**—Sends only InterOp files to BaseSpace Sequence Hub, which allows you to monitor the run remotely.

- d Log on to BaseSpace Sequence Hub or BaseSpace Onsite Sequence Hub with your Myllumina account email and password.
- 3 Select **Browse** to navigate to a preferred output folder location.
- 4 Confirm that the thumbnail setting is **Save All Thumbnails**.
The software automatically saves all thumbnail images. A thumbnail is a sampling of images from many tiles in each column of tiles, or swath, combined in one image.
- 5 Select **Next**.

Flow Cell Setup Screen

The Flow Cell Setup screen records information about the flow cell used for the run. All fields are required.

- 1 Scan or enter the flow cell ID (barcode number) of the flow cell to be sequenced.
- 2 Select the appropriate flow cell type, **HiSeq 3000/4000 PE** or **HiSeq 3000/4000 SR**.
- 3 Enter an experiment name to appear on each screen and help identify the run in progress.
- 4 Enter a user name.
- 5 Select **Next**.

Advanced Screen

- 1 **[Optional]** Select the **Confirm First Base** checkbox.
A first base report is generated automatically for each run after cycle 2 and placed in the root level of the run folder. Selecting this option allows you to confirm the first base report before proceeding with the run. Otherwise, the run continues without showing the confirmation dialog box.
- 2 **[Optional]** From the flow cell image, select lanes to remove from the run.
All lanes are included by default. PhiX alignment is performed automatically for all lanes.
- 3 Select **Next**.

Recipe Screen

A recipe is generated automatically from the information entered on the Recipe screen.

- 1 Select an Index Type option:
 - ▶ **No Index**—Performs a non-indexed single-read or paired-end run.
 - ▶ **Single Index**—Performs a single-read or paired-end run with one indexing read.
 - ▶ **Dual Index**—Performs a single-read or paired-end run with two indexing reads.
 - ▶ **Custom**—Performs a single-read or paired-end run with a custom number of cycles for index reads.
- 2 Enter the number of cycles for Read 1 and Read 2, if applicable.



NOTE

The number of cycles performed in a read is one more cycle than the number of cycles analyzed. For example, to perform 125 cycles for Read 1, enter 126.

- 3 If you selected the **Custom** indexing option, enter up to 10 cycles for each Index Read.



NOTE

Read lengths do not need to match.

- 4 Confirm the following autopopulated chemistry settings.
 - ▶ **SBS: HiSeq 3000/4000 SBS Kit**—Shows the SBS chemistry used for Read 1 and Read 2.
 - ▶ **Index: HiSeq 3000/4000 Sequencing Primer** or **HiSeq 3000/4000 Dual Index Sequencing Primer**—Shows the chemistry used for index reads.
 - ▶ **PE turnaround: HiSeq 3000/4000 PE** or **HiSeq 3000/4000 PE Dual Index**—Shows the chemistry used for paired-end resynthesis.
- 5 **[Optional]** Select the **Use Existing Recipe** checkbox to use a custom recipe.

Sample Sheet Screen

Sample sheets are optional unless you use BaseSpace Sequence Hub to perform data analysis.

- 1 Select **Browse** to locate the sample sheet.
- 2 Select **Next**.

Reagents Screen

The Reagents screen records information about the reagent kit used for the run.

- 1 Scan or enter the SBS reagent kit barcode ID.
- 2 For paired-end runs, scan or enter the cluster kit ID.
- 3 Select the SBS reagent kit for the run:
 - ▶ Select **300 Cycles** for a 300-cycle kit. Cycles remaining defaults to 325.
 - ▶ Select **150 Cycles** for a 150-cycle kit. Cycles remaining defaults to 174.
 - ▶ Select **50 Cycles** for a 50-cycle kit. Cycles remaining defaults to 74.
 - ▶ Select **Custom** for a partial kit or multiple 50-cycle kits. In the Cycles Remaining field, enter the number of SBS cycles that reagents are expected to last.



NOTE

The Cycles Remaining field is populated automatically based on the SBS kit ID. The software counts down the number of cycles entered. When the cycles are low, the software prompts you to load fresh reagents.

- 4 Select **Prime SBS Reagents** to prime reagents.
- 5 Select **Next**.

Review Screen

- 1 Review run parameters from the Review screen.
- 2 Select **Next** to proceed or **Back** to change parameters.

Load and Prime Reagents

After entering run parameters, load SBS, indexing, and paired-end reagents for the run, and then prime reagents through the fluidics system. The software guides you through these steps in a series of screens on the Pre-Run Setup tab.

Load SBS Reagents

- 1 Invert each bottle to mix.

**CAUTION**

Mix and load HCM last, after all other reagents are loaded, to prevent cross-contamination. Always discard your gloves and replace them with a new pair after handling HCM.

- 2 Replace the cap on each bottle with a funnel cap.
- 3 Open the reagent compartment door.
- 4 Raise the sippers for the SBS reagent rack as follows.
 - a Pull the sipper handle towards you and then raise it.
 - b Release the handle into the slot on the top end of the groove. Make sure that the handle rests securely in the slot.
- 5 Slide the reagent rack out of the reagent compartment using the rack handle.
- 6 Place each bottle into the rack in the associated numbered position. Make sure that the conical end of the bottle rests in the indentation on the base of the rack.

Position	Reagent	Description
1	HIM	HT Incorporation Mix
2	PW1	25 ml PW1 or laboratory-grade water
3	HSM	HT Scan Mix
4	HB1	HT SBS Buffer 1
5	HB2	HT SBS Buffer 2
6	HB2	HT SBS Buffer 2
7	HCM	HT Cleavage Mix
8	HB2	HT SBS Buffer 2

- 7 Put on a new pair of powder-free latex gloves.
- 8 Slide the rack into the reagent compartment, aligning the rack with the raised guide on the compartment floor.
- 9 Lower the sippers into the SBS reagent bottles as follows.
 - a Pull the sipper handle towards you and then lower it.
 - b Inspect the sippers to make sure that they do not bend as they lower into the funnel caps.
 - c Release the handle into the slot on the bottom end of the groove.

Load Indexing and Paired-End Reagents

- 1 Invert each bottle to mix.
- 2 Raise the sippers for the paired-end reagent rack as follows.
 - a Pull the handle towards you and raise it.
 - b Release the handle into the slot on the top end of the groove. Make sure that the handle rests securely in the slot.
- 3 Slide the rack out of the reagent compartment using the rack handle.
- 4 If you are performing a non-indexed single-read run, skip step 5 and load each position with a 15 ml conical tube filled with 10 ml PW1 or laboratory-grade water.

- Remove reagent tube caps and place each tube into the rack in the associated numbered position or matching label color.

Table 1 Paired-End Flow Cell

Position	Reagent	Description
10	HRM	HT Resynthesis Mix
11	HLM2	HT Linearization Mix 2
12	PW1	10 ml PW1 or laboratory-grade water
13	HAM	HT Amplification Mix
14	HPM	HT Amp Premix
15	HDR	HT Denaturation Mix (contains formamide)
16	HP11	Primer Mix – Read 2
17	HP14*	Indexing Primer Mix
18	PW1	10 ml PW1 or laboratory-grade water
19	PW1	10 ml PW1 or laboratory-grade water

*HP14 is required for indexed runs only. If HP14 is not used, load a 15 ml conical tube filled with 10 ml PW1 or laboratory-grade water.

Table 2 Single-Read Flow Cell

Position	Reagent	Description
10	HRM*	HT Resynthesis Mix
11	PW1	10 ml PW1 or laboratory-grade water
12	PW1	10 ml PW1 or laboratory-grade water
13	PW1	10 ml PW1 or laboratory-grade water
14	PW1	10 ml PW1 or laboratory-grade water
15	HDR	HT Denaturation Mix (contains formamide)
16	PW1	10 ml PW1 or laboratory-grade water
17	HP12	Index 1 Primer Mix
18	PW1	10 ml PW1 or laboratory-grade water
19	PW1	10 ml PW1 or laboratory-grade water

*HRM is required for dual-indexed runs only. If HRM is not used, load a 15 ml conical tube filled with 10 ml PW1 or laboratory-grade water.

- Slide the rack into the compartment, aligning the rack with the raised guide on the compartment floor.
- Lower the sippers into the paired-end reagent tubes as follows.
 - Pull the handle towards you and lower it.
 - Inspect the sippers to make sure that they do not bend as they lower into the tubes.
 - Release the handle into the slot on the bottom end of the groove.
- Select the **PW1 (25 ml) loaded in Position 2** checkbox, and then select **Next**.

Prime Reagents

Steps for priming reagents include loading a priming flow cell, confirming proper flow, and then starting the prime.

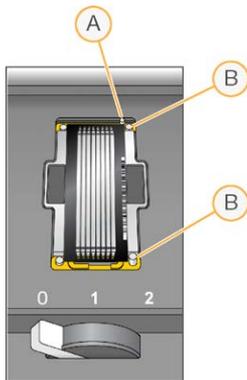
**CAUTION**

Always use a *used* flow cell to prime reagents. You can use the flow cell from a previous run to prime reagents on a subsequent run or for a post-run wash.

Load a Priming Flow Cell

- 1 Scan or enter the ID (barcode number) of the priming flow cell.
- 2 Rinse the priming flow cell with laboratory-grade water. Dry with a lens cleaning tissue or lint-free tissue.
- 3 Clean with alcohol wipes and lens cleaning tissue.
- 4 Place on the flow cell holder with the inlet and outlet ports facing **down** and the barcode on the right. Make sure that the arrow on the left edge of the flow cell, which indicates flow direction, points towards the instrument.
- 5 Gently slide the flow cell towards the top and right guide pins until it stops.

Figure 7 Flow Cell Positioned Against Top and Right Guide Pins



- A Top guide pin
- B Right guide pins

- 6 Remove your hand from the flow cell to prevent alignment drift.
- 7 Slowly move the flow cell lever to position 1 to engage the vacuum and secure the flow cell. When the flow cell lever is blinking green, the vacuum is engaged. If the lever is not green, see *Possible Run Setup Problems on page 32*.
- 8 Wait for about five seconds, and then slowly move the flow cell lever to position 2. When the flow cell lever is solid green, the manifolds are in position and the flow cell is ready.
- 9 Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.

Confirm Proper Flow

Checking for proper flow confirms that the flow cell and gaskets are properly installed and the manifold is engaged.

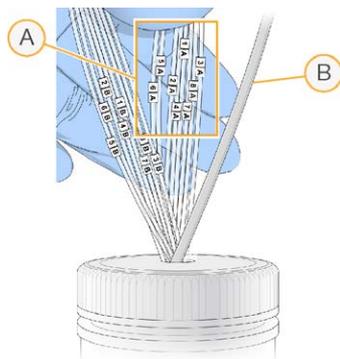
- 1 Select Position **2** from the drop-down list.
- 2 Confirm the following default values:
 - ▶ Volume: **125**
 - ▶ Aspirate Rate: **250**

- ▶ Dispense Rate: **2000**
- 3 Select **Pump**.
 - 4 Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
 - 5 If excessive bubbles are present, do as follows.
 - a Check the gaskets for obstructions.
 - b Reduce the aspirate rate to 100.
 - c Pump another 125 μ l of water to the flow cell.
 - d If problems persist, remove the flow cell, repeat the cleaning steps, and reload the flow cell.
 - 6 Select **Next**.

Position Tubing and Start Prime

- 1 Remove the 8 waste tubes for the appropriate flow cell from the waste container.

Figure 8 Position Tubing



- A Flow cell waste tubes for reagent positions 1–8
- B Condensation pump tubing

- 2 Place each waste tube into a separate empty 15 ml tube. Waste is collected and measured when priming is complete.
- 3 Select **Start Prime**. Monitor priming progress from the Prime screen.
- 4 When priming is complete, measure the waste and confirm that the volume in each tube is 1.75 ml for a total of **14 ml**.
The total is calculated as follows:
 - ▶ 250 μ l for each SBS position except position 2 ($250 \times 7 = 1.75$ ml)
 - ▶ 1.75 ml for each lane ($1.75 \times 8 = 14$ ml)
- 5 Return the waste tubes to the waste container.
- 6 Select **Next**.

Load the Sequencing Flow Cell

Loading the flow cell for sequencing includes removing the priming flow cell, cleaning the flow cell holder, loading the clustered flow cell, and confirming proper flow.

Remove the Used Flow Cell

- 1 Slowly move the flow cell lever to position 1 to disengage the manifolds.
- 2 Slowly move the flow cell lever to position 0 to disengage the vacuum seal and release the flow cell.
- 3 Lift the used flow cell from the flow cell holder.

Clean the Flow Cell Holder

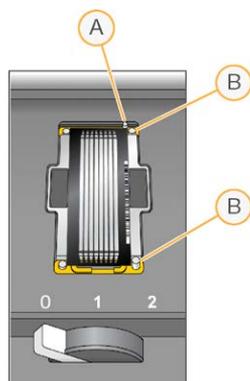
- 1 Put on a new pair of powder-free latex gloves.
- 2 Wipe the surface of the flow cell holder with a lint-free tissue moistened with laboratory-grade water to remove salts.
- 3 Wipe the surface of the flow cell holder with an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol. Do not allow alcohol to drip into the vacuum holes or around the manifolds.
- 4 Dry the stage with a low-lint lab tissue, if necessary.
- 5 Inspect the flow cell holder to make sure that it is free of lint and the vacuum holes are free of obstructions.

Figure 9 Inspect Vacuum Holes



Load the Sequencing Flow Cell

- 1 Place the flow cell on the flow cell holder with the inlet and outlet ports facing **down** and the barcode on the right. Make sure that the arrow on the left edge of the flow cell, which indicates flow direction, points toward the instrument.
- 2 Gently slide the flow cell toward the top and right guide pins until it stops.

Figure 10 Flow Cell Positioned Against Top and Right Guide Pins

- A Top guide pin
- B Right guide pins

- 3 Remove your hand from the flow cell to prevent alignment drift over time.
- 4 Slowly move the flow cell lever to position 1 to engage the vacuum and secure the flow cell. When the flow cell lever is blinking green, the vacuum is engaged. If the lever is not green, see *Possible Run Setup Problems on page 32*.
- 5 Wait about five seconds, and then slowly move the flow cell lever to position 2. When the flow cell lever is solid green, the manifolds are in position and the flow cell is ready for use.
- 6 Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.

Confirm Proper Flow

Checking for proper flow confirms that the flow cell and gaskets are properly installed and the manifold is engaged.

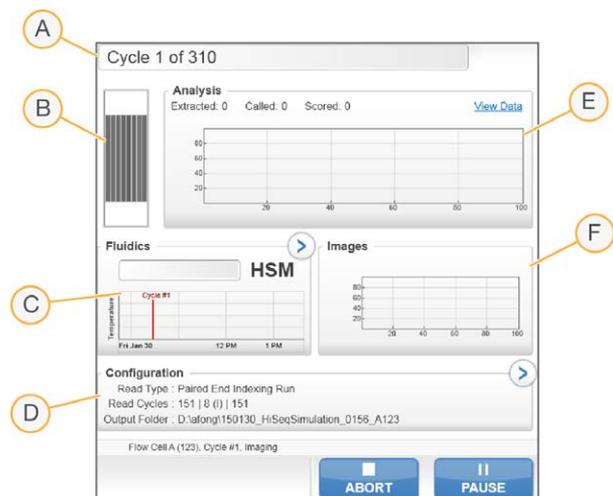
- 1 Select **Position 5** from the drop-down list.
- 2 Enter the following values:
 - ▶ Volume: **250**
 - ▶ Aspirate Rate: **250**
 - ▶ Dispense Rate: **2000**
- 3 Select **Pump**.
- 4 Inspect the flow cell for bubbles passing through the lanes or leaks near the manifolds.
- 5 If excessive bubbles are present, do as follows.
 - a Check the manifold gaskets for obstructions.
 - b Repeat the pump using position 6 to avoid depleting position 5.
 - c Reduce the aspirate rate to 100.
 - d Pump another 250 μ l to the flow cell.
- 6 Select **Next**.
- 7 Make sure that the flow cell lever is green, and then close the flow cell compartment door.
- 8 Make sure that the **Vacuum Engaged** and **Door Closed** checkboxes are selected, and then select **Next**.

- 9 Select **Start** to begin the sequencing run.

Monitor the Run

- 1 Monitor run metrics from the Run Overview screen.

Figure 11 Run Overview Screen



- A **Progress bar**—Monitor how many cycles have been completed.
- B **Flow cell image**—Monitor imaged lanes.
- C **Fluidics graph**—Expand the fluidics section to monitor chemistry steps.
- D **Run Configuration**—Review parameters of current run.
- E **Analysis graph**—Monitor quality scores by cycle.
- F **Images graph**—Monitor intensities by cycle. One thumbnail image is shown for each swath scanned. No other images appear on the software interface.

First Base Report

If you opted to confirm first base during run setup, the first base confirmation dialog box opens automatically after imaging of the second cycle is complete. The run pauses at this step.

- 1 Review the First Base Report from the confirmation dialog box.
- 2 If the results are satisfactory, select **Continue**.

View Run Metrics

When run metrics are available, Sequencing Analysis Viewer (SAV) opens automatically and displays them. Metrics appear in the form of plots, graphs, and tables. For more information, see the *Sequencing Analysis Viewer User Guide (document # 15020619)*.

- 1 To view updated metrics, select **Refresh** at any time during the run.

Unload Reagents

- 1 When the run is complete, open the reagent compartment door.
- 2 Raise the sippers for the appropriate SBS rack and paired-end rack as follows.

- a Pull the sipper handle outward.
 - b Raise the sipper handle while pulling it outward.
 - c Release the sipper handle into the slot on the top end of the groove. Make sure that the sipper handle rests securely in the slot.
- 3 Slide each reagent rack out of the reagent compartment using the rack handles.
 - 4 Remove each bottle from each reagent rack.

**WARNING**

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Perform a Water Wash

A water wash is required after each sequencing run to wash the system and check fluidics. A maintenance wash is an optional alternative to the post-run water wash. For instructions, see *Perform a Maintenance Wash on page 26*.

If the instrument has been idle for one day or more, perform a water wash before beginning a new sequencing run.

- 1 From the Welcome screen, select **Wash | Water**.
- 2 Select **Yes** to wash paired-end reagent positions, and then select **Next**.
- 3 Load the instrument with laboratory-grade water:
 - a Fill eight SBS bottles with 250 ml laboratory-grade water.
 - b Fill 10 PE tubes with 12 ml laboratory-grade water.

**NOTE**

Wash bottles and tubes are typically replaced every six months, although the water is replaced about every week.

- 4 Make sure that a used flow cell is loaded. Load a used flow cell, if necessary.
- 5 Select **Next**.
- 6 Perform a fluidics check:
 - a Select solution 2 from the drop-down list.
 - b Accept the default pump values.
 - c Select **Pump**.
 - d Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
- 7 Remove the waste tubes for the appropriate flow cell from the waste container.
- 8 Bundle the waste tubes with parafilm. Keep all the ends even.
- 9 Place the bundled tube ends into a 250 ml bottle.
- 10 Select **Next** to start the water wash.

Positions	Approximate Run Time
Eight SBS positions	20 minutes
Eight SBS positions and 10 paired-end positions	60 minutes

- 11 When the wash is complete, measure the delivered volume.

Positions	Total Delivered Volume	Per Lane Delivered Volume
Eight SBS positions	32 ml	4 ml
Eight SBS positions and 10 paired-end positions	72 ml	9 ml

- 12 Unwrap the waste tubes and return them to the waste bottle.

Quick Format the Output and Scratch Drives

After data transfer completes, perform a quick format of the output (O:\) and scratch (S:\) drives. A quick format clears the drive for a subsequent run without removing important system or instrument maintenance files.

Before a run can begin, a minimum of 2 TB is required for a run length of 2 x 151. If disk space drops below the safe threshold during the run, the software pauses the run and places the flow cell in a safe state. After disk space is made available, the run resumes automatically.



NOTE

Instrument maintenance logs are stored on the C:\ drive. Therefore, it is safe to perform a quick format of the O:\ and S:\ drives during an instrument wash.

- 1 From Windows, open Computer to show the list of drives on the computer.
- 2 Right-click the O:\ drive and select **Format**.
- 3 From the Format dialog box, select the **Quick Format** checkbox.
- 4 Select **Start**.
- 5 Repeat steps 1–4 to clear the S:\ drive.

Chapter 5 Maintenance

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Introduction

Maintenance procedures ensure continued instrument performance.

- ▶ Shut down or idle the instrument during periods of inactivity.
- ▶ Supplement the water wash performed at the end of a run with regular maintenance washes to maintain fluidics.

Regular instrument washes maintain instrument performance by flushing the fluidics system and preventing salt accumulation and cross-contamination of reagents.

Preventive Maintenance

Illumina recommends that you schedule a preventive maintenance service each year. If you are not under a service contract, contact your Territory Account Manager or Illumina Technical Support to arrange for a billable preventive maintenance service.

Perform a Maintenance Wash

Perform a maintenance wash when prompted by the software every 10 days or optionally after a run. A maintenance wash takes about 90 minutes and follows one of two workflows, depending on whether ProClin 300 is available:

- ▶ **Tween 20 and ProClin 300 wash**—Washes the system with a user-prepared solution of Tween 20 and ProClin 300. See *Tween 20 and ProClin 300 Maintenance Wash* on page 26.
- ▶ **Tween 20 wash**—Washes the system with a user-prepared solution of Tween 20 and can require a water wash. See *Tween 20 Maintenance Wash* on page 28.

When the Load Gasket screen appears before a maintenance wash, the gaskets in the front manifold and back manifold must be replaced before starting the wash.

Tween 20 and ProClin 300 Maintenance Wash

Prepare Maintenance Wash Solution

Prepare 5 liters of maintenance wash solution for use with one instrument. The solution can be stored for up to 30 days at room temperature and used up to three times during this period.

Dispose of the wash solution in accordance with the governmental safety standards for your region.

- 1 Adding the water first, combine the following volumes to dilute Tween 20:
 - ▶ Laboratory-grade water (225 ml)
 - ▶ Tween 20 (25 ml)These volumes result in approximately 10% Tween 20.
- 2 Place a stir bar in an empty carboy that is at least 6 liters.
- 3 Adding the water first, combine the following volumes in the carboy:
 - ▶ Laboratory-grade water (750 ml)

- ▶ 10% Tween 20 (250 ml)
- ▶ ProClin 300 (1.5 ml)

These volumes result in a solution that is approximately 2.5% Tween 20 and 0.15% ProClin 300.

- 4 Mix thoroughly on a stir plate.
- 5 Add 4 liters laboratory-grade water.
These volumes result in a solution that is approximately 0.5% Tween 20 and 0.03% ProClin 300.
- 6 Continue stirring until thoroughly mixed.
- 7 Set aside in a closed container at room temperature.

Tween 20 and ProClin 300

- 1 From the Welcome screen, select **Wash | Maintenance**.
- 2 If you are using fresh maintenance wash solution, load the instrument with solution as follows.
 - a Fill eight SBS bottles with 250 ml fresh wash solution.
 - b Fill 10 PE tubes with 12 ml fresh wash solution.
 - c Assign each bottle and tube to a reagent rack position. Maintain these assignments for each subsequent wash to prevent cross-contamination from reagent present on the sippers.
- 3 If you stored maintenance wash solution from a previous run, load the instrument with solution as follows.
 - a Replenish the stored solution and invert to mix. Replenish no more than two times after the original use.
 - b Load the bottles and tubes into the assigned reagent rack positions.

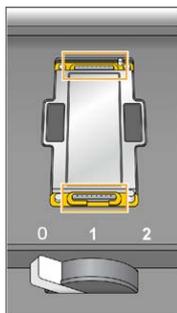


NOTE

Monthly replacement of wash bottles and tubes is typically sufficient.

- 4 Empty the waste bottle.
- 5 Select **Next**.
- 6 Remove the flow cell from the flow cell stage and set it aside.
- 7 Put on a new pair of powder-free latex gloves.
- 8 Apply light pressure to one side of the front gasket until the other side lifts. Use tweezers to grasp and remove the gasket. Repeat to remove the rear gasket.

Figure 12 Remove Used Manifold Gaskets



- 9 Place a new gasket in each slot on the front end and back end of the flow cell holder. Press lightly into position.

- 10 Reload the flow cell that you removed to install the new gaskets.
- 11 Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.
- 12 Perform a fluidics check using the default pump values:
 - a Select solution 2 from the drop-down list.
 - b Select **Pump**.
 - c Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
 - d If you see a constant stream of bubbles, replace the gasket and repeat the fluidics check.
- 13 Remove the waste tubes for the appropriate flow cell from the waste container.
- 14 Bundle the eight waste tubes with parafilm. Keep the tubes ends even.
- 15 Place the bundled tube ends into a 250 ml bottle.
- 16 Select **Next** to start the wash.
- 17 When the wash is complete, select **Return to Start**.
- 18 Measure the delivered volume.

Positions	Delivered Volume
Eight SBS positions	74 ml
10 paired-end positions	52 ml
All positions	15.75 ml per lane

**NOTE**

All bottles and tubes are filled to capacity to make sure that the sippers are rinsed. However, the delivered volume for each position varies so the bottles and tubes contain different volumes when the wash is complete.

- 19 Unwrap the waste tubes and return them to the waste container.

Tween 20 Maintenance Wash

Prepare Maintenance Wash Solution

Always prepare fresh wash solution for a Tween 20 maintenance wash. Prepare 5 liters of maintenance wash solution. This volume is sufficient to wash both sides of one instrument.

Dispose of wash solution in accordance with the governmental safety standards for your region.

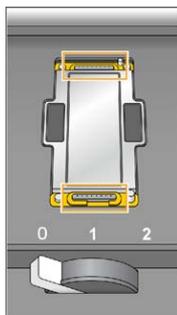
- 1 Adding the water first, combine the following volumes to dilute Tween 20:
 - ▶ Laboratory-grade water (225 ml)
 - ▶ Tween 20 (25 ml)
 These volumes result in approximately 10% Tween 20.
- 2 Place a stir bar in an empty carboy that is at least 6 liters.
- 3 Adding the water first, combine the following volumes in the carboy:
 - ▶ Laboratory-grade water (750 ml)
 - ▶ 10% Tween 20 (250 ml)
 These volumes result in a solution that is approximately 2.5% Tween 20.
- 4 Mix thoroughly on a stir plate.

- 5 Add 4 liters laboratory-grade water to result in a solution that is approximately 0.5% Tween 20.
- 6 Continue stirring until thoroughly mixed.
- 7 Immediately proceed to setting up the wash.

Tween 20 Wash

- 1 From the Welcome screen, select **Wash | Maintenance**.
- 2 Load the instrument with fresh maintenance wash solution as follows.
 - a Fill eight SBS bottles with 250 ml fresh wash solution.
 - b Fill 10 PE tubes with 12 ml fresh wash solution.
- 3 Empty the waste bottle.
- 4 Select **Next**.
- 5 Remove the flow cell from the flow cell stage and set it aside.
- 6 Put on a new pair of powder-free latex gloves.
- 7 Apply light pressure to one side of the front gasket until the other side lifts. Use tweezers to grasp and remove the gasket. Repeat to remove the rear gasket.

Figure 13 Remove Used Manifold Gaskets



- 8 Place a new gasket in each slot on the front end and back end of the flow cell holder. Press lightly into position.
- 9 Reload the flow cell that you removed to install the new gaskets.
- 10 Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.
- 11 Perform a fluidics check using the default pump values:
 - a Select solution 2 from the drop-down list.
 - b Select **Pump**.
 - c Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
 - d If you see a constant stream of bubbles, replace the gasket and repeat the fluidics check.
- 12 Remove the waste tubes for the appropriate flow cell from the waste container.
- 13 Bundle the eight waste tubes with parafilm. Keep the tubes ends even.
- 14 Place the bundled tube ends into a 250 ml bottle.
- 15 Select **Next** to start the wash.

- 16 When the wash is complete, select **Return to Start**.
- 17 Measure the delivered volume.

Positions	Delivered Volume
Eight SBS positions	74 ml
10 paired-end positions	52 ml
All positions	15.75 ml per lane

**NOTE**

All bottles and tubes are filled to capacity to make sure that the sippers are rinsed. However, the delivered volume for each position varies so the bottles and tubes contain different volumes when the wash is complete.

- 18 Unwrap the waste tubes and return them to the waste container.

Water Wash

If the instrument is going to be idle for more than five days after the Tween 20 wash, perform a water wash. The water wash rinses Tween 20 from the fluidics system.

- 1 From the Welcome screen, select **Wash | Water Wash**.
- 2 Load the instrument with laboratory-grade water as follows.
 - a Fill eight SBS bottles with at least 20 ml laboratory-grade water.
 - b Fill 10 PE tubes with 10 ml laboratory-grade water.

**CAUTION**

Do not reuse the water, bottles, or tubes used for the Tween 20 wash. The water might be contaminated with reagents present on the sippers.

- 3 Load the bottles and tubes onto the instrument in the appropriate reagent rack.
- 4 Select **Next** to start the wash.
- 5 When the wash is complete, measure the delivered volume.

Positions	Delivered Volume
Eight SBS positions	32 ml
Eight SBS positions and 10 paired-end positions	72 ml

- 6 Unwrap the waste tubes and return them to the waste container.

Idle the Instrument

Use the following instructions to prepare the instrument to sit idle for up to 10 days. For durations longer than 10 days, shut down the instrument instead.

- 1 Perform a maintenance wash to flush the system.
- 2 Leave the flow cell on the flow cell stage with the flow cell lever in position 2. Leave the manifolds in the raised position.
- 3 Load 10 ml laboratory-grade water in each position in the reagent racks, and then lower the sippers.
- 4 Before using the instrument, perform a water wash.

Shut Down the Instrument

Use the following procedure to prepare fluidics safely and shut down the system. Shut down the instrument only if you do not plan to use it within the next 10 days or more. If you plan to use the instrument within the next 10 days, idle it instead.

- 1 Perform a maintenance wash to flush the system.
- 2 Remove the flow cell from the flow cell stage.
- 3 Wipe the surface of the flow cell holder using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol.



CAUTION

Do not allow alcohol to drip into the vacuum holes or around the manifolds. Use a low-lint lab tissue to dry the stage, if necessary.

- 4 Load 10 ml laboratory-grade water in each position in the reagent racks, and then lower the sippers.
- 5 Turn off the instrument.
- 6 To restart the instrument:
 - a Load water in all reagent positions.
 - b Turn on the instrument.
 - c Perform a water wash.

Appendix A Troubleshooting

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Log File

The log file lists any errors that occurred in the control software. Use this file for troubleshooting purposes.

To access the log file, select **Menu | Tools | Show Log** from the Welcome screen.

Possible Run Setup Problems

Problem	Possible Cause	Action
The software did not initialize.	The software was unable to initialize internal hardware devices.	Close the error message and then relaunch the instrument software. If the problem persists, restart the instrument computer. If you are going to restart the computer, first shut down the instrument to make sure the DoNotEject drive is recognized correctly. If the problem persists after restarting the instrument computer, shut down the instrument, wait a minimum of 60 seconds, and then restart the instrument.
Flow cell lever is orange.	The flow cell did not seat properly. The vacuum did not seal. Manifolds did not raise.	Remove the flow cell and repeat the cleaning steps. Make sure that the gaskets are present and well-seated. Reload the flow cell. If the preceding steps do not work, try replacing the gaskets, and then reload the flow cell.
Flow cell lever is blinking orange.	Vacuum is being provided but is inadequate.	Remove the flow cell and repeat the cleaning steps. Make sure that the gaskets are present and well-seated. Reload the flow cell. If the preceding steps do not work, try replacing the gaskets, and then reload the flow cell.
Flow cell lever is blinking green.	Vacuum pressure is good.	Switch flow cell lever to position 2.
Poor fluid delivery.	Potential bubbles in the system.	Reposition the flow cell and confirm that the holes are facing down . Look for white precipitate around the gaskets. If precipitate is present, replace the gaskets. Always replace gaskets before an instrument maintenance wash. Confirm that the sipper assemblies are fully lowered and each sipper is in contact with the reagents.
Loss of registration in Read 1 characterized by no intensities and 0% clusters passing filter in a portion of the flow cell. Percentage of clusters passing filter abruptly decreases from tile 1 (inlet) to tile 28 (outlet).	The flow cell did not seat properly.	If the run has not completed paired-end turnaround, stop the run and rehyb the flow cell. Before restarting the run, see Load the Sequencing Flow Cell on page 20 to ensure proper seating of the flow cell. If the run has completed paired-end turnaround, set up a new run with a new flow cell.

Perform a Fluidics Check

Perform a fluidics check during instrument installation and when troubleshooting fluidics issues.

- 1 Select **Check** on the Welcome screen.
- 2 Scan or enter the wash flow cell ID (barcode number) of the priming flow cell. Make sure to use a *used* flow cell for this step.
- 3 Load the used flow cell onto the instrument.
- 4 Fill eight SBS bottles with PW1 or laboratory-grade water, and load the bottles onto the SBS reagent rack.
- 5 Select solution 2 from the drop-down list.
- 6 Confirm the following default values:
 - ▶ Volume: **250**
 - ▶ Aspirate Rate: **250**
 - ▶ Dispense Rate: **2000**
- 7 Select **Pump**.
- 8 Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
- 9 If excessive bubbles are present:
 - a Check the manifold gaskets for obstructions.
 - b Reduce the aspirate rate to 100.
 - c Pump another 250 µl of water to the flow cell.

Pause or End a Run on the HiSeq 4000

Ending a run does not provide the option to save data or resume the run. Pausing a run might be necessary to check run components or set up a run on the adjacent flow cell.

Pause a Run

Pause a run to check run components, such as reagent volumes, as necessary. Under normal operation, pausing a run is not necessary.

RTA2 resumes automatically after a paused run is resumed, so the run can resume without loss of data. For more information, see [Real-Time Analysis on page 36](#).

- 1 From the Run Overview screen, select **Pause | Normal Pause**.
- 2 Select **Yes** to confirm the command.

The software completes the current chemistry or imaging command and places the flow cell in a safe state.
- 3 Select **Resume** to resume the run.

Change Reagents During a Run

If you started the run with a partial volume of reagents, use the Change Reagents feature to pause the run and replenish reagents.

**NOTE**

Priming is not required.

- 1 From the Run Overview screen, select **Pause** to open the pause menu.
- 2 Select **Change Reagents**.
- 3 Select **Yes** to confirm the pause command.
The software completes the current chemistry or imaging command, places the flow cell in a safe state, and opens the Reagents screen.
- 4 Enter the following parameters:
 - ▶ The reagent kit ID for the new reagents.
 - ▶ The number of cycles the reagents are expected to last.
- 5 Select **Next** to proceed to loading reagents.

End a Run

If RTA2 is terminated, the software does not resume processing and run data are not saved. Therefore, a run cannot resume after it has been stopped.

**CAUTION**

Ending a run on the HiSeq 4000 is *final*.

- 1 To end the run, select **Abort**. Confirm or cancel the command.
- 2 Confirming the commands opens the Welcome screen.
- 3 Proceed to post-run procedures.

**NOTE**

If a run is stopped during Read 1, it is possible to perform primer rehybridization on the cBot. After primer rehybridization, start a new run on the HiSeq 4000 to sequence the flow cell.

Stagger Runs on Flow Cell A and Flow Cell B

- 1 Select **Pause | Normal Pause**.
- 2 Wait for the software to complete the current chemistry or imaging step.
The system is automatically placed in a safe state.
- 3 Confirm that the run is successfully paused.
When a run is paused, the Resume button is present.
- 4 Set up the new run.
- 5 After loading the new flow cell for the new run, close the compartment door.
- 6 Select **Start** to start the new run.
- 7 On the adjacent flow cell, select **Resume** to resume the paused run.
The software automatically controls chemistry and imaging processes on both flow cells.

Possible Read 1 Primer Rehybridization

If Read 1 metrics indicate low cluster numbers, low intensities, or other concerns, you can perform a Read 1 primer rehybridization to rescue the flow cell. Read 1 primer rehybridization is performed on the cBot and does not damage clusters on the flow cell.

Read 1 primer hybridization on a HiSeq 4000 patterned flow cell requires the following Illumina consumables:

- ▶ HiSeq 3000/4000 cBot Multi-Primer Rehybridization Kit (catalog # GD-305-2001)
- ▶ HiSeq cBot Manifold (catalog # SY-401-2015)

For more information, see *Read 1 Primer Rehyb on a HiSeq 3000/4000 Flow Cell (document # 15058794)*.

Appendix B Real-Time Analysis

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Real-Time Analysis Overview

The HiSeq 4000 uses an implementation of Real-Time Analysis software called RTA2. RTA2 runs on the instrument computer and extracts intensities from images, performs base calling, and assigns a quality score to the base call. RTA2 and the control software communicate through a web HTTP interface and shared memory files. If RTA2 is terminated, processing does not resume and run data are not saved.



NOTE

Demultiplex performance is not calculated, so the Index tab in Sequencing Analysis Viewer (SAV) is not populated.

Input Files

RTA2 requires the following input files:

- ▶ Tile images contained in local system memory.
- ▶ **RunInfo.xml**, which the control software generates automatically at the beginning of the run. From this file, RTA2 reads the run name, number of cycles, whether a read is indexed, and the number of tiles on the flow cell.
- ▶ **RTA.exe.config**, which is a software configuration file in XML format.

RTA2 receives commands from the control software that include information about the location of **RunInfo.xml** file and if an optional output folder is specified.

Output Files

Images for each channel are passed in memory to RTA2 as tiles. From these images, RTA2 produces primary output as a set of quality-scored base call files and filter files. Other files support generation of primary output files.

- ▶ **Base call files**—For each tile that is analyzed, one compressed base call (*.bcl) file is generated for each tile per cycle. The base call file contains the base call and associated quality score.
- ▶ **Filter files**—Each tile produces filter information that is included in one filter (*.filter) file for each tile over the whole run. The filter file specifies whether clusters pass filter.
- ▶ **Cluster location files**—One cluster location (s.locs) file contains the X,Y coordinates for every cluster on the flow cell.

Primary output files are used for subsequent data analysis. Use the bcl2fastq conversion software for demultiplexing and FASTQ conversion. To convert data from the HiSeq 4000, use bcl2fastqv2.16, or later. For the current software version and download information, see the HiSeq 4000 support page on the Illumina website.

RTA2 provides real-time metrics of run quality stored as InterOp files. InterOp files are binary files containing tile, cycle, and read-level metrics, and required to view metrics in Sequencing Analysis Viewer. For viewing metrics generated by RTA2, use SAV v1.10.2, or later.

For details about each output file, see [Sequencing Output Files on page 41](#).

Error Handling

RTA2 creates log files and writes them to the RTALogs folder. Errors are recorded in an error file in *.tsv file format.

The following log and error files are transferred to the final output destination at the end of processing:

- ▶ *GlobalLog*.tsv summarizes important run events.
- ▶ *LaneNLog*.tsv lists processing events for each lane.
- ▶ *Error*.tsv lists errors that occurred during a run.
- ▶ *WarningLog*.tsv lists warnings that occurred during a run.

Data Transfer

Throughout the run, RTA2 requests data transfer from Run Copy Service, the software that manages the transfer to the specified output folder location. If BaseSpace Sequence Hub is used, the BaseSpace Broker manages the transfer of data to BaseSpace Sequence Hub. If the network connection is interrupted, RTA2 continues processing and writes data locally. Data transfer resumes when the connection is restored.

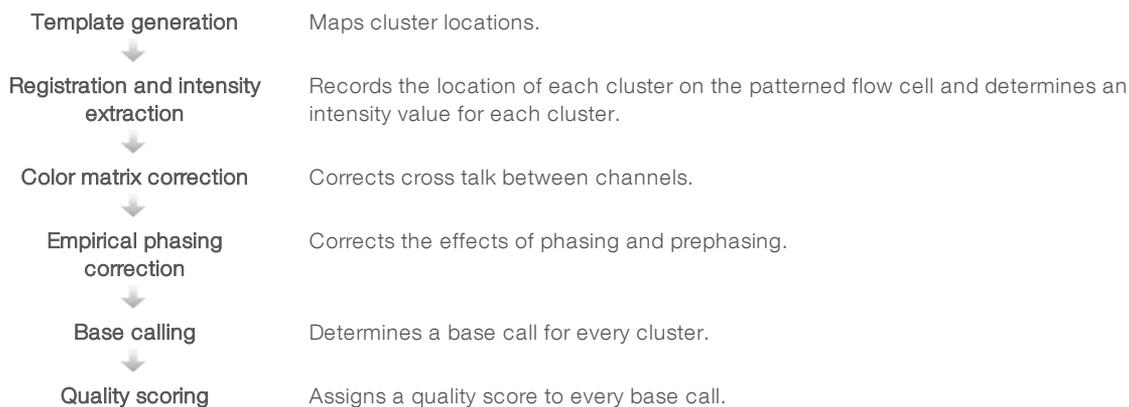


NOTE

Make sure that your network connection meets the minimum requirements for sending run data to BaseSpace Sequence Hub. For more information, see the site prep guide.

After processing is complete, RTA2 creates a marker file called RTAComplete.txt. Data transfer completes after this file is generated. A sensor indicator at the bottom of the screen shows transfer status. For details, see [Activity and Sensor Indicators on page 5](#).

Real-Time Analysis Workflow



Template Generation

Template generation defines the position of each cluster in a tile using X and Y coordinates. The template is used as a reference for the subsequent step of registration and intensity extraction.

Because of the array on the patterned flow cell, cluster positions are predetermined per the number of rows and columns, and the distance between nanowells. For more information, see [Patterned Flow Cell on page 8](#).

Cluster positions are written to one cluster location (s.locs) file for the entire run.

Registration and Intensity Extraction

Registration and intensity extraction begin after the template of cluster positions is generated.

- ▶ Registration transforms the template locations of clusters to the location on the image in each of the four color channels.
- ▶ Intensity extraction determines an intensity value for each cluster in the template for a given image.

If registration fails for any images in a cycle, no base calls are generated for that tile in that cycle. Use SAV to examine thumbnail images and identify images that failed registration.

Color Matrix Correction

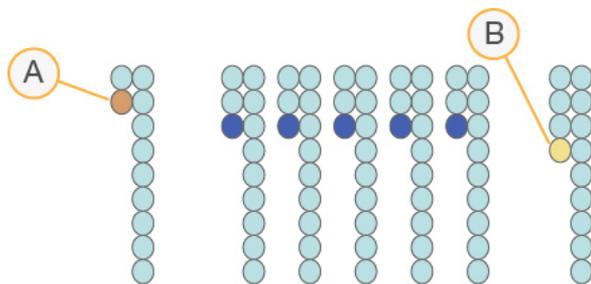
After registration and intensity extraction, RTA2 corrects for cross talk between channels. Cross talk occurs when a cluster shows intensity in the C channel and some intensity also shows in the A channel, for example. Using a 4 x 4 color matrix, RTA2 generates matrix-corrected intensities with reduced or no cross talk, and balances differences in overall intensity between color channels.

Empirical Phasing Correction

During the sequencing reaction, each DNA strand in a cluster extends by one base per cycle. Phasing and prephasing occurs when a strand becomes out of phase with the current incorporation cycle.

- ▶ Phasing occurs when a base falls behind.
- ▶ Prephasing occurs when a base jumps ahead.

Figure 14 Phasing and Prephasing



- A Read with a base that is phasing
- B Read with a base that is prephasing.

RTA2 corrects the effects of phasing and prephasing using the empirical phasing correction algorithm, which maximizes the data quality at every cycle throughout the run.

Base Calling

After raw intensities are corrected for cross talk, phasing, and prephasing, the channel with the brightest intensity is the call for that cluster in that cycle. Base calling on the HiSeq 4000 using RTA2 begins after cycle 3.

Base calling determines a base (A, C, G, or T) for every cluster of a given tile at a specific cycle. Base calls are saved to base call (*.bcl) files, which are binary files with 1 byte per call and quality score. Each base call file contains the base call and the base call quality score. To make a base call, clusters must first pass the chastity filter. Clusters that do not pass filter or cannot be called because they are off-image or failed image registration are labeled no-calls. No-calls are represented as (N).

Clusters Passing Filter

During the first 25 cycles of Read 1, the chastity filter removes low-quality clusters from analysis results. Clusters pass filter if no more than one base call has a chastity value below 0.6 in the first 25 cycles. Chastity is defined as the ratio of the brightest base intensity divided by the sum of the brightest and the second brightest base intensities. The percentage of clusters passing filter is represented in analysis reports as %PF. The HiSeq 4000 patterned flow cell has an ordered array of clusters. Empty wells with no clusters and polyclonal wells where more than one sequence exists are included in the raw cluster count, but do not pass filter. Therefore, the ordered array on a patterned flow cell yields a relatively low percentage of clusters passing filter.

Figure 15 Empty and Polyclonal Wells (Included in Raw Cluster Count)

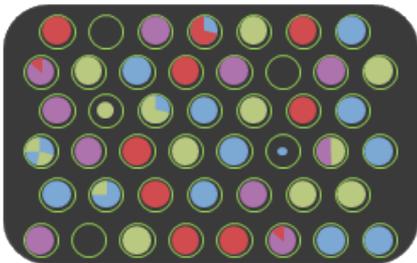
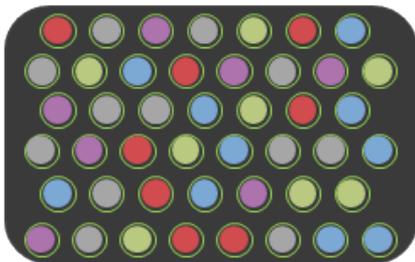


Figure 16 Wells With Non-PF Clusters (Shown in Gray)



Quality Scoring

A quality score, or Q-score, is a prediction of the probability of an incorrect base call. A higher Q-score implies that a base call is higher quality and more likely to be correct.

The Q-score is a compact way to communicate small error probabilities. Q(X) represents quality scores, where X is the score. The following table shows the relationship between the quality score and error probability.

Q-score Q(X)	Error Probability
Q40	0.0001 (1 in 10,000)
Q30	0.001 (1 in 1,000)
Q20	0.01 (1 in 100)
Q10	0.1 (1 in 10)



NOTE

Quality scoring is based on a modified version of the Phred algorithm.

Quality scoring calculates a set of predictors for each base call, and then uses the predictor values to look up the Q-score in a quality table. Quality tables are created to provide optimally accurate quality predictions for runs generated by a specific configuration of sequencing platform and version of chemistry.

After the Q-score is determined, results are recorded in the base call files.

Q-Score Binning

RTA2 groups quality scores into specific ranges, or bins, and assigns a value to each range. Q-score binning significantly reduces storage space requirements without affecting accuracy or performance of downstream applications.

Q-score binning contributes to the efficiency of analysis processes and data transfer requirements associated with the high throughput of the HiSeq 4000. The resulting *.bcl file is smaller because the compression algorithms are able to compress the file more effectively. Less data are written to the instrument computer and transferred to a network location, making the file copy faster.

Appendix C Output Files

Output Files

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Sequencing Output Files

File Type	File Description, Location, and Name
Base call files	Each tile analyzed is included in a base call file that contains the base call and encoded quality score. Data\Intensities\BaseCalls\L00[X] —Files are stored in per cycle folders for each lane. s_[Lane]_[Tile].bcl.gz , where lane is the single-digit lane number and tile is the four-digit tile number. Base call files are compressed using gzip compression.
Cluster location files	For each tile, 1 cluster location file contains the XY coordinates for every cluster. Cluster location files are the result of template generation. Data\Intensities —One file for the run is stored in the Intensities folder. s.locs
Filter files	The filter file specifies whether a cluster passed filters. Filter files are generated at cycle 26 using 25 cycles of data. Data\Intensities\BaseCalls\L00[X] —Files are stored in one folder for each lane and tile. s_[lane]_[tile].filter
InterOp files	Binary reporting files used for Sequencing Analysis Viewer. InterOp files are updated throughout the run. InterOp folder
Real-Time Analysis configuration file	Created at the beginning of the run, the Real-Time Analysis configuration file lists settings for the run. [Root folder] RTAConfiguration.xml
Run information file	Lists the run name, number of cycles in each read, whether the read is indexed, and the number of swaths and tiles on the flow cell. The run info file is created at the beginning of the run. [Root folder] RunInfo.xml
Thumbnail files	A thumbnail image for each channel and tile in each swath at every cycle during imaging. Thumbnail_Images\L00[X]\C[X.1] —Files are stored in one folder for each lane and one subfolder for each cycle. s_[lane]_[tile]_[channel].jpg —Tile is represented with a four-digit number that indicates surface, swath, and tile. See <i>Tile Numbering</i> on page 42.

Output Folder Structure

 **Config**—Configuration settings for the run.

 **Data**

 **Intensities**

 **BaseCalls**

 **L00[X]**—Base call files for each lane, aggregated in one file per cycle.

 **s.locs**

 **Images**

 **Focus**

 **L00[X]**—Focus images for each lane.

-  **InterOp**—Binary files used by Sequencing Analysis Viewer.
-  **Logs**—Log files describing operational events.
-  **Recipe**—Run-specific recipe file named with reagent cartridge ID.
-  **RTALogs**—Log files describing RTA2 events.
-  **Thumbnail_Images**—Thumbnail images of nine locations from a subset of tiles, generated for each cycle and base.
-  RTAConfiguration.xml
-  RunInfo.xml
-  RunParameters.xml

Run Folder Name and Path

The run folder is the root folder for output from a sequencing run. During run setup, the software prompts you to enter the path for the run folder. By default, the folder is named using the following format:

YYMMDD_<Computer Name>_<Run Number>_<Flow Cell Side><Flow Cell ID>

Example: 110114_SN106_0716_A90095ACXX

The run number increments by one each time the instrument performs a sequencing run. The flow cell side (A or B) and the flow cell ID entered during the run setup steps are appended to the run folder name.

The run folder is written to the output path specified during run setup. The temporary run folder for flow cell A is written to the S: drive and the temporary run folder for flow cell B is written to the O: drive.

Tile Numbering

The HiSeq 3000/4000 patterned flow cell is imaged in 112 tiles on each lane, top and bottom, for each cycle. Each of the eight lanes has two swaths with 28 tiles per swath. Tiles are numbered according to position.



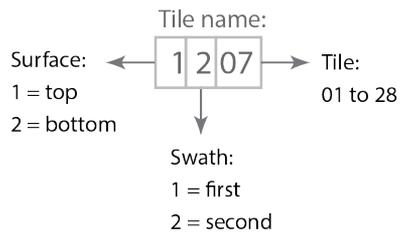
NOTE

A swath is a column of tiles within a lane of the flow cell.

The tile name is a four-digit number that represents the position on the flow cell.

- ▶ The first digit represents the surface:
 - ▶ 1 is for top
 - ▶ 2 is for bottom
- ▶ The second digit represents the swath:
 - ▶ 1 is for the first swath
 - ▶ 2 is for the second swath
- ▶ The last two digits represent the tile, 01 through 28. Tile numbering starts with 01 at the output end of the flow cell through 28 at the input end.

Figure 17 Tile Numbering



This example indicates a tile from the top surface of the flow cell, the second swath, and the seventh tile.

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

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North America	+1.800.809.4566	
Australia	+1.800.775.688	
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Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
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Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
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New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
South Korea	+82 80 234 5300	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.



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