# HiSeq 2000

## System Guide

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# Revision History

| Document | Date | Description of Change |
|---|---|---|
| Document # 15011190 v03 | May 2019 | Re-added SBS Kits for Dual–Indexed Sequencing information which was previously moved to the support site.<br>Corrected HP14 reagent information back to HP12.<br>Minor text edits. |
| Document # 15011190 v02 | March 2018 | Added cBot as a compatible cluster instrument.<br>Added Custom Protocol Selector to Additional Resources.<br>Added information about the Illumina Proactive monitoring service in the View and Send Instrument Data section.<br>Noted approximate frequency for renewing wash bottles and tubes.<br>Updated the name of analysis software to BaseSpace Sequence Hub.<br>Updated reagent information to replace HP12 with HP14.<br>Updated instructions for starting the instrument:<br>• Removed the default user name and password required to log on to the operating system. Illumina recommends using site-specific credentials.<br>• Wait for the system to load before logging on to the operating system, not after.<br>• Increased the duration for instrument devices to be configured and DoNotEject to initialize from 1 minute to 3 minutes.<br>• Noted that hard drives must be empty for proper operation.<br>Removed recommendation to perform a maintenance wash instead of a water wash after a sequencing run.<br>Corrected the run folder naming format to include the flow cell side.<br>Updated the maintenance wash procedure. |
| Document # 15011190 v01 | October 2015 | Added instructions for preparing SBS, indexing, and paired-end reagents.<br>Added recommendation for annual preventive maintenance service. |
| Part # 15011190 Rev. V | October 2014 | Replaced NaOH maintenance wash with Tween 20 and ProClin 300 maintenance wash including information about preparing, storing, and disposing of maintenance wash solution.<br>Updated descriptions of maintenance wash and water wash to specify that a water wash is required after a run.<br>Added workflow, input and output files, error handling, and quality scoring descriptions to Real-Time Analysis chapter.<br>Updated VWR catalog # for alcohol wipes to 95041-714.<br>Updated URL for Safety Data Sheets (SDS) to support.illumina.com/sds.html. |
| Part # 15011190 Rev. U | April 2014 | Updated software descriptions to HCS v2.2, which includes the HiSeq v4 high output mode, removal of the control lane option, default Q-score binning, and the option to use different indexing schemes in each lane.<br>Added the HiSeq v4 workflow for use with HiSeq v4 chemistry.<br>Added calculation for total SBS priming volume. |

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| Document | Date | Description of Change |
|---|---|---|
| Part # 15011190 Rev. T | November 2013 | Removed reagent preparation instructions. For reagent prep instructions including information about various sequencing primers, see the documentation for the associated kit. |
| Part # 15011190 Rev. R | May 2013 | Replaced the following reagents:<br>• RMR for RMX |
| Part # 15011190 Rev. P | January 2013 | Error correction; removed HiSeq 2500 info from Performing a Sequencing Run sections. |
| Part # 15011190 Rev. N | November 2012 | Revised to support HCS 2.0. |
| Part # 15011190 Rev. M | July 2012 | Listed primer requirements for TruSeq HT libraries and Nextera libraries.<br>Added list of supported dual-indexed libraries.<br>Updated run setup workflow for dual-indexed libraries to specify TruSeq Dual Index Sequencing Primer Box regardless of which sequencing primers are used for the run. |
| Part # 15011190 Rev. L | April 2012 | Changed the run setup workflow: manifold gaskets must be replaced before performing the instrument maintenance wash instead of before loading the clustered flow cell.<br>Added the following information:<br>• Overviews of single-indexed and dual-indexed sequencing runs.<br>• Description of dual-indexed libraries.<br>• Example ICB volumes for dual-indexed runs.<br>• Reagent preparation instructions for SBS, paired-end, and indexing reagents, including sequencing primers required for dual-indexed libraries.<br>• Emphasis on using the correct version of TruSeq Dual Index Sequencing Primer Box based on flow cell type. |
| Part # 15011190 Rev. K | October 2011 | Updated the following information:<br>• Instructions for splitting ICB<br>• HP3 volumes for indexed and paired-end runs<br>• Number of SBS kits required for dual-indexed runs |
| Part # 15011190 Rev. J | October 2011 | Updated software descriptions to HCS v1.5.<br>Added instructions for setting up and performing dual-indexed runs for Nextera libraries. |
| Part # 15011190 Rev. H | June 2011 | Added instructions to load 25 ml of laboratory-grade water to SBS position 2 for the automatic post-run rinse. |

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| Document | Date | Description of Change |
|---|---|---|
| Part # 15011190 Rev. G | April 2011 | Updated software descriptions to HCS v1.4.<br>Added the following information:<br>• Description of flow cell orientation check<br>• Approximate run durations for HCS v1.4<br>• Version compatibility of software and reagent kit versions for various flow cell types<br>• Information about reagents in the TruSeq SBS Kit v3, including description of SBS v3 alternative workflow for loading reagents<br>• Instructions for idling the instrument<br>Updated the following information:<br>• Reagent positions with SBS v3 reagent names<br>• Instructions for shutting down the instrument<br>• Increased the amount of water required for the instrument maintenance wash |
| Part # 15011190 Rev. F | February 2011 | Added description for performing staggered runs before HCS v1.3.<br>Corrected reagent name. |
| Part # 15011190 Rev. E | January 2011 | Updated software descriptions to HCS v1.3.<br>Updated instructions for resuming a run. |
| Part # 15011190 Rev. D | May 2010 | Corrected Sequencing Kit box 1 and box 2 contents.<br>Updated volumes in the preparation of Multiplexing Rd2 Seq Primer for use on HiSeq. |
| Part # 15011190 Rev. C | April 2010 | Corrected LFN thawing instructions and volumes in the multiplexing reagent prep instructions. |
| Part # 15011190 Rev. B | March 2010 | Updated software descriptions to HCS v1.0.<br>Added instructions for changing reagents during a run, and stopping, pausing, and resuming a run.<br>Updated reagent preparation instructions and added instructions for allocating reagents for 101 cycles. |
| Part # 15011190 Rev. A | February 2010 | Initial release. |

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# Chapter 1 Overview

## Introduction

The HiSeq® 2000 system provides power and efficiency for large-scale genomics. Innovative engineering combines with proven SBS technology to set new standards in output, simplicity, and cost-effectiveness.

## Features

▶ **Dual-surface imaging**—The HiSeq 2000 uses a 4-camera epifluorescence system with cutting-edge scanning technology to enable dual surface imaging.

▶ **Dual flow cells**—The HiSeq 2000 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 IlluminaCompute, Illumina BaseSpace, or 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.

## 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 2500, 1500, and 2000 Site Prep Guide (document # 15006407)* | Provides specifications for laboratory space, electrical requirements, and environmental considerations. |
| *HiSeq 1500 and HiSeq 2000 Safety and Compliance Guide (document # 1000000000665)* | Provides information about instrument labeling, compliance certifications, and safety considerations. |

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| Resource | Description |
|---|---|
| *HiSeq and GAIIx Systems Denature and Dilute Libraries Guide (document # 15050107)* | Provides instructions for denaturing and diluting prepared libraries for a sequencing run, and preparing a PhiX control. This step applies to most library types. |

Visit the HiSeq 2000 support page on the Illumina website for access to documentation, software downloads, online training, and frequently asked questions.

## Instrument Components

The HiSeq 2000 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
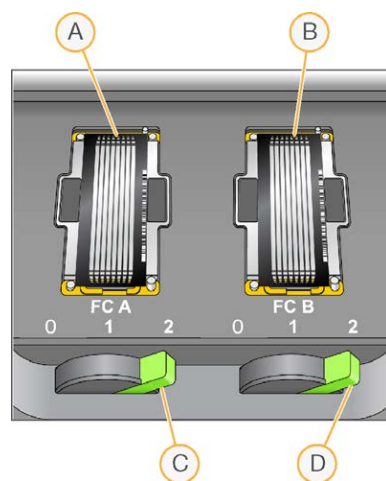


A    Flow cell A
B    Flow cell B
C    Flow cell lever A
D    Flow cell lever B

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
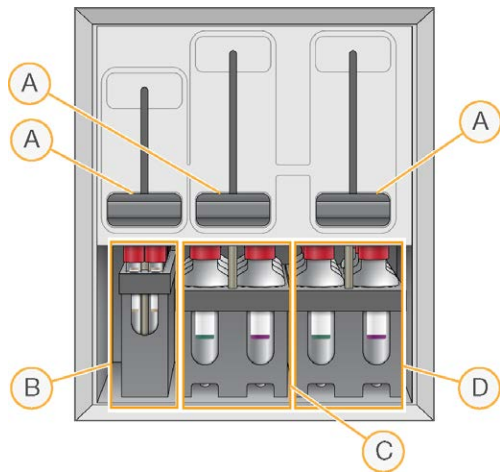


| A | Sipper handles |
|---|---|
| B | Reagent rack for indexing and paired-end reagents |
| C | Reagent rack for SBS reagents for flow cell A |
| D | Reagent rack for SBS reagents for flow cell B |

# HiSeq 2000 Software

Three software applications are installed on the instrument computer:

▶ **HiSeq 2000 control software**—The HiSeq Control Software (HCS) 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 (RTA) performs base calling and assigns a quality score to each base for each cycle. For more information, see *Real-Time Analysis* on page 56.

▶ **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. |

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| 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
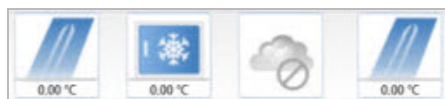


From left to right, sensor indicators represent flow cell A temperature, reagent chiller temperature, data transfer status, BaseSpace Hub cloud status, and flow cell B temperature.

## Available Disk Space

The HiSeq instrument computer has a storage capacity of over 2.7 TB per flow cell. Data from flow cell A is stored on the D: drive, and data from flow cell B is stored on the E: drive.

At the end of each imaging cycle for each lane, the software checks available disk space on the local D: and E: drives. If disk space becomes low, the software pauses the run and places the flow cell in a safe state. Create more space to continue the run, which resumes automatically when sufficient space is available.

## Sequencing Consumables Overview

Performing a run on the HiSeq 2000 requires one SBS kit and one cluster kit. Cluster kits are available in paired-end (PE) and single-read (SR) versions.

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. Each cluster kit includes an accessory kit that provides flow cell gaskets, funnel caps for SBS reagent bottles, and a flow cell storage tube.

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## Reagent Kits for HiSeq v4 Mode

| Kit Name | Catalog # |
| --- | --- |
| HiSeq SBS Kit v4 (250 cycles) | FC-401-4003 |
| HiSeq SBS Kit v4 (50 cycles) | FC-410-4002 |
| HiSeq SR Cluster Kit v4 | GD-401-4001 |
| HiSeq PE Cluster Kit v4 | PE-401-4001 |

## SBS Kits for Dual-Indexed Sequencing

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

▶ One 250-cycle SBS kit provides reagents for up to 275 cycles of sequencing.

▶ One 50-cycle kit provides reagents for up to 75 cycles of sequencing.

▶ For runs greater than a total of 275 cycles, use multiple 50-cycle kits.

The following table lists the number of cycles performed for dual-indexed runs of various lengths. For dual-indexed paired-end runs, 7 chemistry-only cycles are calculated in the total number of cycles.

| Run Type | Run Length | Run Cycles | Total Cycles |
| --- | --- | --- | --- |
| Dual-Indexed Paired-End | 2 x 125 | 126+8+(7)+8+126 | 275 |
| | 2 x 100 | 101+8+(7)+8+101 | 225 |
| | 2 x 75 | 76+8+(7)+8+76 | 175 |
| | 2 x 50 | 51+8+(7)+8+51 | 125 |
| | 2 x 35 | 36+8+(7)+8+36 | 95 |
| Dual-Indexed Single-Read | 1 x 125 | 126+8+8 | 142 |
| | 1 x 75 | 76+8+8 | 92 |
| | 1 x 50 | 51+8+8 | 67 |
| | 1 x 35 | 36+8+8 | 52 |

## Reagent Kits for TruSeq v3 Mode

| Kit Name | Catalog # |
| --- | --- |
| TruSeq SBS Kit v3 (200 cycles) | FC-401-3001 |
| TruSeq SBS Kit v3 (50 cycles) | FC-401-3002 |
| TruSeq SR Cluster Kit v3 | GD-401-3001 |
| TruSeq PE Cluster Kit v4 | PE-401-3001 |

## SBS Kits for Dual-Indexed Sequencing

The following table lists the number of cycles performed for dual-indexed runs of various lengths. For dual-indexed paired-end runs, 7 chemistry-only cycles are calculated in the total number of cycles.

▶ One 200-cycle kit provides sufficient reagents for up to 209 cycles of sequencing.

▶ One 50-cycle kit provides sufficient reagents for 58 cycles of sequencing.

▶ For runs greater than a total of 209 cycles, and to maximize reagent use for other read lengths, use multiple 50-cycle kits.

| Run Type | Run Length | Run Cycles | Total Cycles |
|---|---|---|---|
| Dual-Indexed Paired-End | 2 x 100 | 101+8+(7)+8+101 | 225 |
| | 2 x 92 | 93+8+(7)+8+93 | 209 |
| | 2 x 75 | 76+8+(7)+8+76 | 175 |
| | 2 x 50 | 51+8+(7)+8+51 | 125 |
| | 2 x 35 | 36+8+(7)+8+36 | 95 |
| Dual-Indexed Single-Read | 1 x 100 | 101+8+8 | 117 |
| | 1 x 75 | 76+8+8 | 92 |
| | 1 x 50 | 51+8+8 | 67 |
| | 1 x 35 | 36+8+8 | 52 |

## Sequencing Primers for Nextera Libraries

The Index 1 sequencing primer (HP8) and Read 2 sequencing primer (HP7) provided in the TruSeq v3 cluster kits are not compatible with Nextera libraries. When sequencing Nextera libraries, use the Index 1 sequencing primer (HP12) and Read 2 sequencing primer (HP11) provided in the TruSeq Dual Index Sequencing Primer Box.

Performing a dual-index run on a single-read flow cell requires HP9, which is provided in the SR version of the kit.

| Kit Name | Catalog # |
|---|---|
| TruSeq Dual Index Sequencing Primer Box, SR | FC-121-1003 |
| TruSeq Dual Index Sequencing Primer Box, PE | PE-121-1003 |

# Chapter 2 Getting Started

## Start the HiSeq 2000

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 D:\ and E:\ 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 and LIMS preferences. The Menu Options window provides settings to define the run ID template, default folder locations, whether to send instrument health information to Illumina, and LIMS authentication.

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.

To activate the command to initialize the software manually, select **Menu | Scanner**.

# 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**
   > These locations can be changed on a per run basis.

4  To set the location where temporary files are written during a run, enter a location in the **Default Temp Folder 1** field.

5  To set a location for LIMS sample forms, enter the location in the **Run Setup Folder** field.

6  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.

# 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**.

▶ **Send instrument health information to Illumina to aid technical support** — Select 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.

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▶ Is turned on by default. To opt out of this service, disable the **Send instrument health information to Illumina to aid technical support** 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. |
| Conical tubes, 50 ml, self-standing (optional) | Corning, catalog # 430921 | Storing flow cells. |
| 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. |
| Pipette tips, 200 µl | General lab supplier | Splitting reagent volumes. |
| Pipette tips, 1000 µl | General lab supplier | Splitting reagent volumes. |
| 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. |

# Sequencing in HiSeq v4 Mode

## Introduction

To perform a sequencing run in HiSeq v4 mode on the HiSeq 2000, 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.

Visit the HiSeq 2000 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 52.

### Run Types for HiSeq v4 Chemistry

The following table shows types of sequencing runs and the number of possible cycles for each read when using HiSeq v4 chemistry. Reference this information when setting up the run.

| Run Type | Read 1 Cycles | Index 1 (i7) Read Cycles | Index 2 (i5) Read Cycles | Read 2 Cycles | Total Cycles |
|---|---|---|---|---|---|
| Single-Read, Non-Indexed | ≤ 126 | -- | -- | -- | ≤ 126 |
| Single-Read, Single-Indexed | ≤ 126 | 6 or 7 [1] 8 [2] | -- | -- | ≤ 133 [1] ≤ 134 [2] |
| Single-Read, Dual-Indexed | ≤ 126 | 8 | 8 | -- | ≤ 142 |
| Paired-End, Non-Indexed | ≤ 126 | -- | -- | ≤ 126 | ≤ 252 |
| Paired-End, Single-Indexed | ≤ 126 | 7 [1] 8 [2] | -- | ≤ 126 | ≤ 259 [1] ≤ 260 [2] |
| Paired-End, Dual-Indexed | ≤ 126 | 8 | 7 + 8 [3] | ≤ 126 | ≤ 275 |

[1] Number of cycles for single-indexed libraries

[2] Number of cycles for dual-indexed libraries

[3] The Index 2 Read of a paired-end dual-indexed run includes 7 additional chemistry-only cycles

# Prepare Reagents

Before setting up the run, prepare all reagents for sequencing: SBS reagents, indexing reagents, and paired-end reagents, if applicable. Load all reagents when prompted by the control software. When using HiSeq v4 chemistry, returning to the instrument during the run to load reagents is not necessary.

Reagents can be prepared during cluster generation. For instructions on preparing clustering reagents and performing cluster generation, see the *cBot System Guide (document # 15006165)*.

## Split Reagents

It is possible to split a 250-cycle kit into 2 sets of reagents to perform shorter runs. For more information, see *Split SBS Kits* on page 53.

## Prepare SBS Reagents

SBS reagents are loaded onto the instrument at the beginning of the run. To prepare reagents, use the following instructions to thaw SBS reagents.

### Thaw SBS Reagents

1   Remove CRM, IRM, and USM from -25°C to -15°C storage. Protect IRM from light.

2   Thaw at 2°C to 8°C for about 16 hours.
    Alternatively, thaw IRM and USM in a room temperature deionized water bath for about 90 minutes. Thaw CRM in a *separate* water bath.

> **NOTE**
> Always replace your gloves after handling CRM.

3   Invert each bottle to mix.

4   Set IRM and USM aside on ice. Set CRM aside on ice *separately* to prevent cross-contamination.

5   Use PW1, SB1, SB2, and SB3 directly from storage.

## Prepare Indexing Reagents

Indexing reagents are used during the indexing reads of an indexed sequencing run.

The PE and SR cluster kits include HP12, which is used for Index 1 (i7) Read 1, regardless of flow cell type. Only the SR cluster kit includes HP9, which is required for a dual-indexed run on a single-read flow cell.

| Run Type | Flow Cell Type | Index 1 (i7) | Index 2 (i5) |
|----------|----------------|--------------|--------------|
| Single-indexed paired-end | PE | HP12 | -- |
| Single-indexed single-read | SR | HP12 | -- |
| Dual-indexed paired-end* | PE | HP12 | -- |
| Dual-indexed single-read | SR | HP12 | HP9 |

* Dual-indexed paired-end runs use FRM, a paired-end reagent, for Index 2.

### Thaw Indexing Reagents

1   Remove the following reagents from -25°C to -15°C storage:
    ▶   For all indexed runs on a paired-end flow cell—FDR and HP12

▸ For single-indexed runs on a single-read flow cell—FDR and HP12

▸ For dual-indexed runs on a single-read flow cell—FDR, HP9, and HP12

2 Thaw reagents in a room temperature deionized water bath for about 20 minutes.

## Prepare FDR and HP12

1 Invert tubes to mix.

2 Centrifuge at 1000 rpm for 1 minute.

3 Set aside at room temperature.

## Prepare HP9

1 Invert to mix.

2 Briefly pulse centrifuge to collect droplets.

3 Set aside at room temperature.

## Prepare Paired-End Reagents

Paired-end reagents are used during the Read 2 resynthesis step of a paired-end sequencing run.

> **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 Paired-End Reagents

1 Remove the following reagents from -25°C to -15°C storage:
▸ For non-indexed runs—AMS, FDR, FLM2, FPM, FRM, and HP11
▸ For indexed runs—AMS, FLM2, FPM, FRM, and HP11

2 Thaw reagents in a beaker filled with room temperature water for about 20 minutes.

3 Place AMS, FLM2, and FRM on ice.

## Prepare AMS, FDR, FLM2, FPM, FRM, and HP11

1 Invert to mix.

2 Centrifuge at 1000 rpm for 1 minute.

3 Set aside AMS, FLM2, and FRM on ice.

4 Set aside FDR, FPM, and HP11 at room temperature.

## 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.

## Integration Screen

The Integration screen provides the option to connect the run to BaseSpace Sequence Hub.

1 From the Welcome screen, select **Sequence** | **New Run** to open the Integration screen.

2 **[Optional]** Connect to BaseSpace Sequence Hub as follows.

    a Select **BaseSpace**.
    b Select from the following BaseSpace 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 remote monitoring of the run.
    c Log on to BaseSpace Sequence Hub using your MyIllumina account email and password.

3 **[Optional]** To proceed without connecting to BaseSpace Sequence Hub, select **None**.

4 Select **Next**.

## Storage Screen

1 Select the **Save to an output folder** checkbox, and then select **Browse** to navigate to a preferred network location.
If the run is connected to BaseSpace Sequence Hub for storage and analysis, this step is optional.

2 Select **Zip BCL files** to reduce required storage space.
If the run is connected to BaseSpace Sequence Hub, the **Zip BCL files** option is selected by default.

> **NOTE**
>
> The **Bin Q-Scores** setting is enabled by default to reduce required storage space. This setting groups quality scores over a wider range of values without affecting accuracy or performance.

3 Select from the following Save Auxiliary Files options:
    ▶ **Save All Thumbnails**—Saves all thumbnails images. A thumbnail is a sampling of images from many tiles in each column of tiles, or swath, combined in 1 thumbnail image.
    ▶ **Save Tile Thumbnails**—Saves tile thumbnails. Tile thumbnails represent a single tile rather than a sampling of tiles in a swath.

4 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.
The flow cell ID determines flow cell type and reagent compatibility.

2 Confirm that the flow cell type is **HiSeq Flow Cell v4**.

3 Enter an experiment name to appear on each screen and help identify the run in progress.

4 Enter a user name, and then select **Next**.

## Advanced Screen

1 **[Optional]** Select the **Confirm First Base** checkbox.

A first base report is generated automatically for each run. Selecting this option opens the first base report before the run proceeds.

2    [Optional] Clear the **Align to PhiX** checkbox of any lane that does not contain PhiX.
     Alternatively, select lanes on the flow cell image to add or remove lanes for PhiX alignment.
     By default, all lanes are selected for alignment.

> **NOTE**
> With HCS v2.2 and RTA v1.18, a dedicated control lane is not required or an option.

3    Select **Next**.

## Recipe Screen

1    Select from the following Index Type options:
     ▶  **No Index**—Performs a non-indexed single-read or paired-end run.
     ▶  **Single Index**—Performs a single-read or paired-end run with 1 indexing read.
     ▶  **Dual Index**—Performs a single-read or paired-end run with 2 indexing reads.
     ▶  **Custom**—Performs a single-read or paired-end run with a custom number of cycles for index reads.

2    If the Dual Index or Custom option is specified, select a Flow Cell Format, either **Single Read** or **Paired End**.

3    Enter the number of cycles for Read 1 and Read 2, if applicable.
     The number of cycles performed in a read is 1 more cycle than the number of cycles analyzed. For example, to perform 125 cycles for Read 1, enter 126.

4    For the **Custom** indexing option, enter the number of cycles for index reads.

> **NOTE**
> Read lengths do not need to be identical.

5    Confirm the following autopopulated chemistry settings:
     ▶  SBS: HiSeq SBS Kit v4
     ▶  Index: HiSeq v4 Single Index or HiSeq v4 Dual Index
     ▶  PE turnaround: HiSeq PE Cluster Kit v4

6    [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 or perform an indexed run.

> **NOTE**
> HCS v2.2 allows a different indexing scheme in each lane.

1    Select **Browse** to locate the sample sheet.

2    Select **Next**.

## Reagents Screen

The Reagents screen records information about reagent kits used for the run. The reagent kit ID (barcode number beginning with **RGT**) determines reagent kit type and run mode compatibility.

1    Scan or enter the SBS reagent kit ID.

2   For paired-end runs, scan or enter the reagent kit ID for the paired-end cluster kit.

3   Select the SBS reagent kit for the run:
  ▶   Select **250 Cycles** for a 250 cycle kit. Cycles remaining defaults to 275.
  ▶   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**
> For partial kits, the software counts down the number of cycles entered. When the cycles are low, the software prompts for 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 reagent bottle several times to mix.

> **CAUTION**
> Handle the bottle of CRM last, after you have loaded all other reagents, to prevent cross-contamination. Always replace your gloves after handling CRM.

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.

6   Place each reagent bottle onto 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 | IRM | Incorporation Reagent Master Mix |
| 2 | PW1 | 25 ml of PW1 or laboratory-grade water |
| 3 | USM | Universal Scan Mix |

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| Position | Reagent | Description |
|---|---|---|
| 4 | SBS Buffer 1 (SB1) | High Salt Buffer |
| 5 | SBS Buffer 2 (SB2) | Incorporation Wash Buffer |
| 6 | SBS Buffer 2 (SB2) | Incorporation Wash Buffer |
| 7 | CRM | Cleavage Reagent Mix |
| 8 | SBS Buffer 3 (SB3) | Cleavage Buffer |

7    Put on a new pair of powder-free latex gloves.

8    Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

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.

10    Select the **PW1 (25 ml) loaded** checkbox.

## Load Indexing Reagents

1    Make sure that the paired-end rack is not in use on the adjacent flow cell for Read 2 resynthesis, Index 1 (i7) Read preparation, and Index 2 (i5) Read preparation.

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 reagent rack out of the reagent compartment using the rack handle.

4    Remove the caps from each reagent tube and place the tube onto the rack in the associated numbered position or matching label color.

Table 1   Single-Read Flow Cells

| Position | Reagent | Description |
|---|---|---|
| 15 | FDR | Fast Denaturation Reagent (contains formamide) |
| 16 | HP9* | Index Sequencing Primer i5 |
| 17 | HP12 | Index Sequencing Primer i7 |

\* HP9 is required for dual-indexed runs only. If HP9 is not used, load a 15 ml conical tube with 10 ml laboratory-grade water in position 16.

Table 2   Paired-End Flow Cells

| Position | Reagent | Description |
|---|---|---|
| 10 | FRM* | Fast Resynthesis Mix |
| 15 | FDR | Fast Denaturation Reagent (contains formamide) |
| 17 | HP12 | Index Sequencing Primer i7 |

\* Load FRM in position 10 for dual-indexed runs on a paired-end flow cell. FRM is required in position 10 for all paired-end runs regardless of indexing option.

5    If you are performing a paired-end run, skip steps 6–9.

6    Place 15 ml conical tubes filled with 10 ml laboratory-grade water in unused positions on the paired-end

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rack.

7 Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

8 Lower the sippers into the paired-end reagent tubes as follows.

    a   Pull the handle towards you and lower it.
    b   Inspect the sippers to make sure that they do not bend as they lower into the tubes.
    c   Release the handle into the slot on the bottom end of the groove.

9 Select **Next**.

## Load Paired-End Reagents

1 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.

2 Slide the reagent rack out of the reagent compartment using the rack handle.

3 Remove the caps from each reagent tube and place the tube onto the rack in the associated numbered position or matching label color.

| Position | Reagent | Description |
| --- | --- | --- |
| 10 | FRM* | Fast Resynthesis Mix |
| 11 | FLM2 | Fast Linearization Mix 2 |
| 13 | AMS | Fast Amplification Mix |
| 14 | FPM | Fast Amplification Premix |
| 15 | FDR* | Fast Denaturation Reagent (contains formamide) |
| 16 | HP11 | Read 2 Sequencing Primer |

\* If you loaded indexing reagents for a single-index run, FRM is already loaded in position 10. If you loaded indexing reagents for a dual-index run, FRM and FDR are already loaded.

4 Place 15 ml conical tubes filled with 10 ml laboratory-grade water in unused positions on the paired-end rack.

5 Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

6 Lower the sippers into the paired-end reagent tubes as follows.

    a   Pull the handle towards you and lower it.
    b   Inspect the sippers to make sure that they do not bend as they lower into the tubes.
    c   Release the handle into the slot on the bottom end of the groove.

7 Select **Next**.

## Prime Reagents

Steps for priming reagents include loading a priming flow cell, confirming proper flow, and then starting the prime.
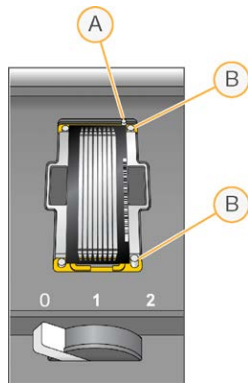
> ⚠ **CAUTION**
> Always use a *used* flow cell to prime reagents. You can use the flow cell from a previous run to prime reagents on a subsequent run or for a post-run wash.

## Load a Priming Flow Cell

1 Rinse the priming flow cell with laboratory-grade water. Dry with a lens cleaning tissue or lint-free tissue.

2 Clean with alcohol wipes and lens cleaning tissue.

3 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.

4 Gently slide the flow cell towards the top and right guide pins until it stops.

Figure 6   Flow Cell Positioned Against Top and Right Guide Pins



A   Top guide pin
B   Right guide pins

5 Remove your hand from the flow cell to prevent alignment drift.

6 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 49.

7 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.

8 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.
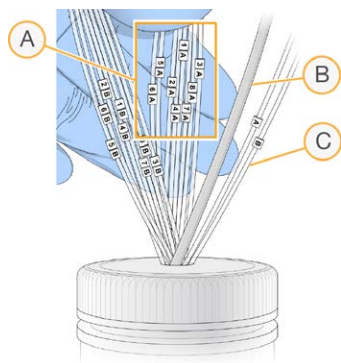
1 Scan or enter the priming flow cell ID (barcode number) of the priming flow cell.

2 Select Position **2** from the drop-down list.

3 Confirm the following default values:
   ▶   Volume: **125**
   ▶   Aspirate Rate: **250**

> ▶ Dispense Rate: **2000**

4　Select **Pump**.

5　Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.

6　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 μl of water to the flow cell.
　　d　If problems persist, remove the flow cell, repeat the cleaning steps, and reload the flow cell.

## Position Tubing and Start Prime

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

**Figure 7** Position Tubing



　A　Flow cell waste tubes for reagent positions 1–8
　B　Condensation pump tubing
　C　Paired-end priming 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 μl for each SBS position except position 2 (250 x 7 = 1.75 ml)
> ▶ 1.75 ml for each lane (1.75 x 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.
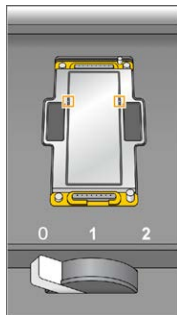
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## Remove the Used Flow Cell

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 8** Inspect Vacuum Holes



## Clean the Flow Cell

1 Remove the clustered flow cell from the container using a pair of plastistats.

2 Rinse the flow cell with laboratory-grade water. Dry with a lens cleaning tissue.

3 Fold an alcohol wipe to approximately the size of the flow cell.

4 Hold the edges of the flow cell with 2 fingers so that the inlet and outlet ports face *up*.

5 Wipe each side of the flow cell using a single sweeping motion. Repeat until clean, refolding the alcohol wipe with each pass.

6 Dry with a dry lens cleaning tissue.

7 Protect the flow cell from dust until you are ready to load it onto the instrument.

## 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 9**  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 49.

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  Confirm the following default 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 µl 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**.

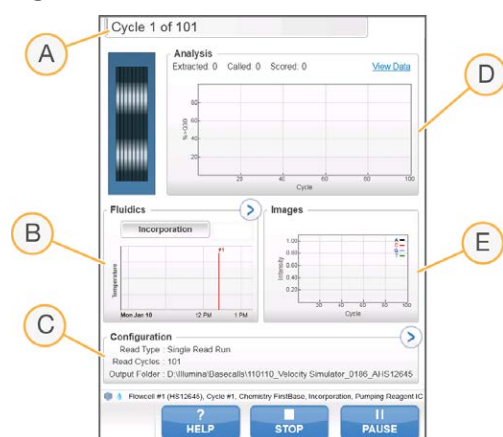9  From the Read Barcode screen, wait for scanning to complete.

The flow cell ID is scanned to confirm a match with the flow cell ID entered at the Flow Cell Setup screen. Scanning takes about three minutes, or seven minutes for a dual-flow cell run.

10  Select **Start** to begin the sequencing run.

## Monitor the Run

1  Monitor run metrics from the Run Overview screen.

**Figure 10**  Run Overview Screen



A  **Progress bar**—Monitor how many cycles have been completed.
B  **Fluidics graph**—Expand the fluidics section to monitor chemistry steps.
C  **Run Configuration**—Review parameters of the current run.
D  **Analysis graph**—Monitor quality scores by cycle.
E  **Images graph**—Monitor intensities by cycle.

## 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 first 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 45*.

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.

# Sequencing in TruSeq v3 Mode

## Introduction

To perform a sequencing run in TruSeq v3 mode on the HiSeq 2000, prepare SBS reagents for Read 1 and indexing reagents before setting up the run. Follow the software prompts to set up the run. Run setup includes entering run parameters, loading and priming reagents, loading the flow cell, and checking fluidics. After the completion of Read 1 and any index reads, prepare and load paired-end reagents and SBS reagents for Read 2.

Visit the HiSeq 2000 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 52.

## Run Types for TruSeq v3 Chemistry

The following table shows types of sequencing runs and the number of possible cycles for each read when using TruSeq v3 chemistry. Reference this information when setting up the run.

| Run Type | Read 1 Cycles | Index 1 (i7) Read Cycles | Index 2 (i5) Read Cycles | Read 2 Cycles | Total Cycles |
|---|---|---|---|---|---|
| Single-Read, Non-Indexed | ≤ 101 | -- | -- | -- | ≤ 101 |
| Single-Read, Single-Indexed | ≤ 101 | 6 or 7 [1] <br> 8 [2] | -- | -- | ≤ 108 [1] <br> ≤ 109 [2] |
| Single-Read, Dual-Indexed | ≤ 101 | 8 | 8 | -- | ≤ 117 |
| Paired-End, Non-Indexed | ≤ 101 | -- | -- | ≤ 101 | ≤ 202 |
| Paired-End, Single-Indexed | ≤ 101 | 7 [1] <br> 8 [2] | -- | ≤ 101 | ≤ 209 [1] <br> ≤ 210 [2] |
| Paired-End, Dual-Indexed | ≤ 101 | 8 | 7 + 8 [3] | ≤ 101 | ≤ 225 |

[1] Number of cycles for single-indexed libraries

[2] Number of cycles for dual-indexed libraries

[3] The Index 2 Read of a paired-end dual-indexed run includes 7 additional chemistry-only cycles

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# Prepare Reagents for Read 1

Before setting up the run, prepare reagents for Read 1 and any index reads. Load prepared reagents when prompted by the control software.

Reagents for Read 1 can be prepared during cluster generation. For instructions on preparing clustering reagents and performing cluster generation, see the *cBot System Guide (document # 15006165)*.

> **NOTE**
> Reagents for Read 2 are prepared during Read 1.

## Split Reagents

It is possible to split a 200-cycle kit into 2 sets of reagents to perform shorter runs. For more information, see *Split SBS Kits* on page 53.

## Prepare SBS Reagents

The steps to prepare SBS reagents include thawing CMR, LFN, and SRE and then preparing ICB with LFN and EDP. Using the 200-cycle SBS kit requires splitting ICB into 2 portions, 1 each for Read 1 and Read 2, during preparation. For dual-indexing, the ICB preparation step includes calculating the appropriate reagent volumes.

**Figure 11** SBS Reagent Preparation Workflow
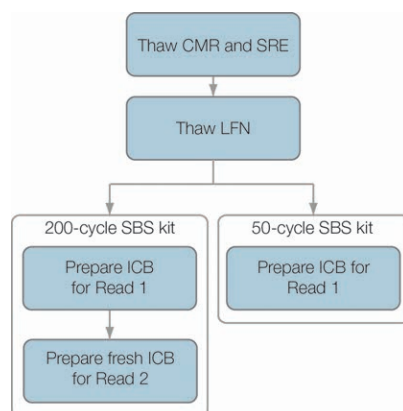


## Thaw SBS Reagents

1 Remove CMR and SRE from -25°C to -15°C storage.

2 Thaw at 2°C to 8°C for about 16 hours.
Alternatively, thaw SRE in a room temperature deionized water bath for about 90 minutes. Thaw CMR in a *separate* water bath.

> **NOTE**
> Always replace your gloves after handling CMR.

3 Invert reagents to mix.

4 Set SRE aside on ice. Set CMR aside on ice *separately* to prevent cross-contamination.

5 Remove LFN from -25°C to -15°C storage:

▶ 50-cycle SBS kit—Remove 1 tube.

▶ 200-cycle SBS kit—Remove 2 tubes.

6 Thaw LFN in a beaker filled with room temperature deionized water for about 20 minutes.

7 Use ICB, SB1, SB2, and SB3 directly from 2°C to 8°C storage.

## Prepare ICB for Read 1 (Non-Index or Single-Index) (50-Cycle Kit)

1 Add the contents of the tube of LFN to the bottle of ICB.

2 Rinse the tube of LFN with ICB to make sure that all LFN is transferred.

3 Add the contents of the tube of EDP to the ICB and LFN solution.

4 Rinse the tube of EDP with the ICB and LFN solution to make sure that all EDP is transferred.

5 Cap the bottle containing EDP, ICB, and LFN and invert to mix.

6 Set aside on ice.

## Prepare ICB for Read 1 (Non-Index or Single-Index) (200-Cycle Kit)

1 Add 47 ml ICB to an empty 250 ml bottle to result in 2 bottles of ICB.

2 Set aside the bottle containing 47 ml ICB at 2°C to 8°C for Read 2.

3 Add the contents of 2 tubes of LFN to the original bottle of ICB.

4 Rinse each LFN tube with ICB to make sure that all LFN is transferred.

5 Add 1.1 ml EDP to the ICB and LFN solution.

6 Return the unused portion of EDP to -25°C to -15°C storage.

7 Cap the bottle containing EDP, ICB, and LFN and invert to mix.

8 Set aside on ice.

## Prepare ICB for Read 1 (Dual-Index)

Use the following instructions to calculate and prepare the volume of ICB required for Read 1 of a dual-indexed run.

1 Measure the following volumes of EDP, ICB, and LFN for every 10 cycles of sequencing to be performed in Read 1.

| Reagent | Volume per 10 Cycles | Storage |
| --- | --- | --- |
| ICB | 4.57 ml | 2°C to 8°C |
| LFN | 0.6 ml | -25°C to -15°C |
| EDP | 0.11 ml | -25°C to -15°C |

2 Transfer the measured volume of ICB to an empty 250 ml bottle.

3 Add the measured volume of LFN to the new bottle of ICB.

4 Rinse each tube of LFN with ICB to make sure that all LFN is transferred.

5 Add the measured volume of EDP to the ICB and LFN solution.

6 Rinse the tube of EDP with the ICB and LFN solution to make sure that all EDP is transferred.

7 Cap the bottle containing EDP, ICB, and LFN and invert to mix.

8    Set aside on ice.

## Example Calculation

A 101-cycle dual-indexed paired-end run has a total of 225 cycles:

▶    124 cycles (101 + 8 + 7 + 8) for Read 1 and the Index Reads

▶    101 cycles for Read 2

| Read 1 and Index Reads (124 Cycles) | Read 2 (101 Cycles) |
|---|---|
| 124 cycles / 10 cycles = 12.4<br>• ICB: 12.4 x 4.57 ml = 56.9 ml<br>• LFN: 12.4 x 0.6 ml = 7.44 ml<br>• EDP: 12.4 x 0.11 ml = 1.36 ml | 101 cycles / 10 cycles = 10<br>• ICB: 10 x 4.57 ml = 45.7 ml<br>• LFN: 10 x 0.6 ml = 6 ml<br>• EDP: 10 x 0.11 ml = 1.1 ml |

Use the same calculation for any dual-indexed run. Base the calculation on the total number of cycles in Read 1 and the Index Reads plus the total number of cycles in Read 2. For dual-indexed paired-end runs, include the 7 chemistry-only cycles in the Index 2 Read.

## Prepare Indexing Reagents

Indexing reagents are used during the indexing reads of an indexed sequencing run. Prepare the reagents that are appropriate for the run type and library type.

| Run Type | Library Type | Index 1 (i7) | Index 2 (i5) |
|---|---|---|---|
| Single-indexed | All libraries except Nextera | HP8 | -- |
|  | Nextera libraries | HP12* | -- |
| Dual-indexed paired-end | All libraries except Nextera | HP8 | RMR |
|  | Nextera libraries | HP12* | RMR |
| Dual-indexed single-read | All libraries except Nextera | HP8 | HP9* |
|  | Nextera libraries | HP12* | HP9* |

* Provided in the TruSeq Dual Index Sequencing Primer Box.

## Thaw Indexing Reagents

1    Remove the following reagents from -25°C to -15°C storage:
   ▶    For all indexed runs—HP3, HP12 or HP8, and HT2
   ▶    For dual-indexed runs on a single-read flow cell—HP9
   ▶    For dual-indexed runs on a paired-end flow cell—RMR

2    Thaw reagents in a beaker filled with room temperature deionized water for about 20 minutes.

## Prepare HT2

1    Invert to mix.

2    Centrifuge at 1000 rpm for 1 minute.

3    Set aside at room temperature.

## Prepare HP3 for Indexed Runs

1    Invert to mix, and then pulse centrifuge.

2   Combine the following volumes of HP3 and PW1:

▶   For single-indexed runs—Transfer 3325 µl PW1 to an empty 15 ml conical tube, and then add 175 µl HP3.

▶   For dual-indexed runs—Transfer 3800 µl PW1 to an empty 15 ml conical tube, and then add 200 µl HP3.

3   Invert to mix.

4   Centrifuge at 1000 rpm for 1 minute.

5   Set aside at room temperature.

## Prepare HP8 or HP12

1   Invert to mix.

2   Centrifuge at 1000 rpm for 1 minute.

3   Set aside at room temperature.

## Prepare RMR

1   Invert to mix.

2   Centrifuge at 1000 rpm for 1 minute. *Do not vortex.*

3   Set aside on ice.

## Prepare HP9

1   Invert to mix.

2   Briefly pulse centrifuge to collect droplets.

3   Set aside at room temperature.

## 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.

## Integration Screen

The Integration screen provides the option to connect the run to BaseSpace Sequence Hub.

1   From the Welcome screen, select **Sequence | New Run** to open the Integration screen.

2   **[Optional]** Connect to BaseSpace Sequence Hub as follows.

a   Select **BaseSpace**.

b   Select from the following BaseSpace 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 remote monitoring of the run.

c   Log on to BaseSpace Sequence Hub using your MyIllumina account email and password.

3    **[Optional]** To proceed without connecting to BaseSpace Sequence Hub, select **None**.

4    Select **Next**.

## Storage Screen

1    Select the **Save to an output folder** checkbox, and then select **Browse** to navigate to a preferred network location.
     If the run is connected to BaseSpace Sequence Hub for storage and analysis, this step is optional.

2    Select **Zip BCL files** to reduce required storage space.
     If the run is connected to BaseSpace Sequence Hub, the **Zip BCL files** option is selected by default.

> **NOTE**
>
> The **Bin Q-Scores** setting is enabled by default to reduce required storage space. This setting groups quality scores over a wider range of values without affecting accuracy or performance.

3    Select from the following Save Auxiliary Files options:
     ▶ **Save All Thumbnails**—Saves all thumbnails images. A thumbnail is a sampling of images from many tiles in each column of tiles, or swath, combined in 1 thumbnail image.
     ▶ **Save Tile Thumbnails**—Saves tile thumbnails. Tile thumbnails represent a single tile rather than a sampling of tiles in a swath.

4    Select **Next**.

## Flow Cell Setup Screen

The Flow Cell Setup screen records information about the flow cell used for the run.

1    Scan or enter the flow cell ID (barcode number) of the flow cell to be sequenced.
     The flow cell ID determines flow cell type and reagent compatibility.

2    Confirm that the flow cell type is **HiSeq Flow Cell v3**.

3    Enter an experiment name to appear on each screen and help identify the run in progress.

4    Enter a user name, and then select **Next**.

## Advanced Screen

1    **[Optional]** Select the **Confirm First Base** checkbox.
     A first base report is generated automatically for each run. Selecting this option opens the first base report before proceeding with the run.

2    **[Optional]** Clear the **Align to PhiX** checkbox of any lane that does not contain PhiX.
     Alternatively, select lanes on the flow cell image to add or remove lanes for PhiX alignment.
     By default, all lanes are selected for alignment.

> **NOTE**
>
> With HCS v2.2 and RTA v1.18, a dedicated control lane is not required or an option.

3    **[Optional]** Select **Keep Intensity Files** for later reanalysis or custom processing.

> **NOTE**
>
> Selecting this option significantly increases the size of the data output folder. Saving intensity files is not required for on-instrument analysis.

4    Select **Next**.

## Recipe Screen

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

1    Select from the following Index Type options:
   ▶   **No Index**—Performs a non-indexed single-read or paired-end run.
   ▶   **Single Index**—Performs a single-read or paired-end run with 1 indexing read.
   ▶   **Dual Index**—Performs a single-read or paired-end run with 2 indexing reads.
   ▶   **Custom**—Performs a single-read or paired-end run with a custom number of cycles for index reads.

2    If the Dual Index or Custom option is specified, select a Flow Cell Format, either **Single Read** or **Paired End**.

3    Enter the number of cycles for Read 1 and Read 2, if applicable.
   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.

4    For the **Custom** indexing option, enter the number of cycles for index reads.

   > **NOTE**
   > Read lengths do not need to be identical.

5    Confirm the following autopopulated chemistry settings:

   a   SBS: TruSeq SBS Kit v3
   b   Index: TruSeq Multiplex Sequencing Primer Box or TruSeq Dual Index Sequencing Primer Box
   c   PE turnaround: TruSeq PE Cluster Kit v3

6    **[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 or perform an indexed run.

   > **NOTE**
   > HCS v2.2 allows a different indexing scheme in each lane.

1    Select **Browse** to locate the sample sheet.

2    Select **Next**.

## Reagents Screen

The Reagents screen records information about reagent kits used for the run. The reagent kit ID (barcode number beginning with **RGT**) is used to determine reagent kit type and run mode compatibility.

1    Scan or enter the SBS reagent kit ID.

2    For paired-end runs, scan or enter the reagent kit ID for the paired-end cluster kit.

3    Select the SBS reagent kit for the run:
   ▶   Select **200 Cycles** for a 200 cycle kit. Cycles remaining defaults to 209.
   ▶   Select **50 Cycles** for a 50 cycle kit. Cycles remaining defaults to 59.
   ▶   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**
> For partial kits, the software counts down the number of cycles entered. When the cycles are low, the software prompts for 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   Replace the cap on each reagent bottle with a funnel cap.

> **CAUTION**
> Handle the bottle of CMR last, after you have loaded all other reagents, to prevent cross-contamination. Always replace your gloves after handling CMR.

2   Open the reagent compartment door.

3   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.

4   Slide the reagent rack out of the reagent compartment.

5   Place each reagent bottle onto 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 | ICB | Incorporation Mix |
| 2 | PW1 | 25 ml of PW1 or laboratory-grade water |
| 3 | SRE | Scan Mix Reagent |
| 4 | SBS Buffer 1 (SB1) | High Salt Buffer |
| 5 | SBS Buffer 2 (SB2) | Incorporation Wash Buffer |
| 6 | SBS Buffer 2 (SB2) | Incorporation Wash Buffer |
| 7 | CMR | Cleavage Mix Reagent |
| 8 | SBS Buffer 3 (SB3) | Cleavage Buffer |

6   Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

7   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.

8   Select the **PW1 (25 ml) loaded** checkbox.

## Load Indexing Reagents

1   Make sure that the paired-end rack is not in use on the adjacent flow cell for Read 2 resynthesis, Index 1 (i7) Read preparation, and Index 2 (i5) Read preparation.

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 reagent rack out of the reagent compartment using the rack handle.

4   Remove the caps from each reagent tube and place the tube onto the rack in the associated numbered position or matching label color.

Table 3   Single-Indexed Run

| Position | Reagent | Description |
| --- | --- | --- |
| 17 | HP8 or HP12 | Index 1 (i7) Sequencing Primer Mix |
| 18 | HP3 | Denaturation Solution |
| 19 | HT2 | Wash Buffer |

Table 4   Dual-Indexed Run on a Single-Read Flow Cell

| Position | Reagent | Description |
| --- | --- | --- |
| 16 | HP9 | Index 2 (i5) SR Sequencing Primer Mix |
| 17 | HP8 or HP12 | Index 1 (i7) Sequencing Primer Mix |
| 18 | HP3 | Denaturation Solution |
| 19 | HT2 | Wash Buffer |

Table 5   Dual-Indexed Run on a Paired-End Flow Cell

| Position | Reagent | Description |
| --- | --- | --- |
| 10 | RMR | Resynthesis Mix |
| 17 | HP8 or HP12 | Index 1 (i7) Sequencing Primer Mix |
| 18 | HP3 | Denaturation Solution |
| 19 | HT2 | Wash Buffer |

5   Place 15 ml conical tubes filled with 10 ml laboratory-grade water in unused rack positions.

6   Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

7   Lower the sippers into the paired-end reagent tubes as follows.

    a   Pull the handle towards you and lower it.

    b   Inspect the sippers to make sure that they do not bend as they lower into the tubes.

c   Release the handle into the slot on the bottom end of the groove.

8   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   Rinse the priming flow cell with laboratory-grade water. Dry with a lens cleaning tissue or lint-free tissue.

2   Clean with alcohol wipes and lens cleaning tissue.

3   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.

4   Gently slide the flow cell towards the top and right guide pins until it stops.

**Figure 12**   Flow Cell Positioned Against Top and Right Guide Pins



A   Top guide pin
B   Right guide pins

5   Remove your hand from the flow cell to prevent alignment drift.

6   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 49.

7   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.

8   Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.

## Confirm Proper Flow

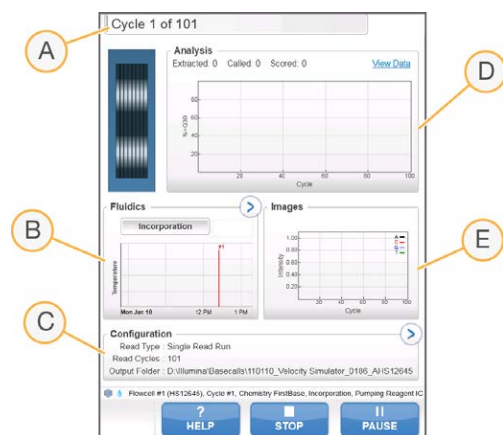Checking for proper flow confirms that the flow cell and gaskets are properly installed and the manifold is engaged.

1   Scan or enter the priming flow cell ID (barcode number) of the priming flow cell.

2   Select Position **2** from the drop-down list.

3   Confirm the following default values:
    ▶   Volume: **125**
    ▶   Aspirate Rate: **250**
    ▶   Dispense Rate: **2000**

4   Select **Pump**.

5   Inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.

6   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 µl of water to the flow cell.
    d   If problems persist, remove the flow cell, repeat the cleaning steps, and reload the flow cell.

## Position Tubing and Start Prime

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

**Figure 13**   Position Tubing



A   Flow cell waste tubes for reagent positions 1–8
B   Condensation pump tubing
C   Paired-end priming 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 µl for each SBS position except position 2 (250 x 7 = 1.75 ml)

▶ 1.75 ml for each lane (1.75 x 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 14** Inspect Vacuum Holes



### Clean the Flow Cell

1 Remove the clustered flow cell from the container using a pair of plastistats.

2 Rinse the flow cell with laboratory-grade water. Dry with a lens cleaning tissue.

3 Fold an alcohol wipe to approximately the size of the flow cell.

4 Hold the edges of the flow cell with 2 fingers so that the inlet and outlet ports face *up*.

5 Wipe each side of the flow cell using a single sweeping motion. Repeat until clean, refolding the alcohol wipe with each pass.

6    Dry with a dry lens cleaning tissue.

7    Protect the flow cell from dust until you are ready to load it onto the instrument.

## 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 15**   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 49.

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    Confirm the following default 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.

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

    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 µl 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    From the Read Barcode screen, wait for scanning to complete.
The flow cell ID is scanned to confirm a match with the flow cell ID entered at the Flow Cell Setup screen. Scanning takes about three minutes, or seven minutes for a dual-flow cell run.

10  Select **Start** to begin the sequencing run.

## Monitor the Run

1    Monitor run metrics from the Run Overview screen.

**Figure 16**  Run Overview Screen



A    **Progress bar**—Monitor how many cycles have been completed.
B    **Fluidics graph**—Expand the fluidics section to monitor chemistry steps.
C    **Run Configuration**—Review parameters of the current run.
D    **Analysis graph**—Monitor quality scores by cycle.
E    **Images graph**—Monitor intensities by cycle.

## 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 first 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.

# Prepare Reagents for Read 2

Before the completion of Read 1 and any index reads, prepare reagents for Read 2 resynthesis and fresh ICB for Read 2. Load reagents when prompted by the control software.

## Prepare Paired-End Reagents

Paired-end reagents are used during the Read 2 resynthesis step of a paired-end sequencing run.

> **NOTE**
>
> Nextera libraries require HP11, a sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box. All other libraries use HP7.

### Thaw Paired-End Reagents

1   Remove the following reagents from -25°C to -15°C storage:
    ▶   For non-indexed or single-indexed runs—AMX2, APM2, AT2, BMX, HP3, HP7 or HP11, HT2, LMX2, and RMR
    ▶   For dual-indexed runs—AMX2, APM2, AT2, BMX, HP3, HP7 or HP11, HT2, and LMX2
    ▶   For paired-end runs—HP3

2   Thaw reagents in a beaker filled with room temperature deionized water for about 20 minutes.

3   Set aside AMX2, BMX, LMX2, and RMR on ice.

### Prepare AMX2, APM2, AT2, BMX, HP3, HP7, HP11, HT2, and LMX2

1   Invert each tube to mix.

2   Centrifuge at 1000 rpm for 1 minute.

3   Set aside AMX2, BMX, and LMX2 on ice.

4   Set aside APM2, AT2, HP3, HP7, HP11, and HT2 at room temperature.

### Prepare HP3 for Paired-End Runs

1   Invert to mix, and then pulse centrifuge.

2   Transfer 2.85 ml PW1 to an empty 15 ml conical tube, and then add 150 µl HP3.

3   Invert to mix.

4   Centrifuge at 1000 rpm for 1 minute.

5   Set aside at room temperature.

## Prepare RMR

1   Invert to mix.

2   Centrifuge at 1000 rpm for 1 minute. *Do not vortex.*

3   Set aside on ice.

## Prepare ICB for Read 2 (Non-Index or Single-Index)

1   Remove 2 tubes of LFN from -25°C to -15°C storage and thaw in a beaker filled with room temperature deionized water for about 20 minutes.

2   When thawed, add the contents of the 2 tubes of LFN to the bottle containing 47 ml ICB.

3   Rinse each LFN tube with ICB to make sure that all LFN is transferred.

4   Add 1.1 ml EDP to the ICB and LFN solution.

5   Return any unused portion of EDP to -25°C to -15°C storage.

6   Cap the bottle containing EDP, ICB, and LFN and invert to mix.

7   Set aside on ice.

## Prepare ICB for Read 2 (Dual-Index)

Use the following instructions to calculate and prepare the volume of ICB required for Read 2 of a dual-indexed run.

1   Measure the following volumes of EDP, ICB, and LFN for every 10 cycles of sequencing to be performed in Read 2.

| Reagent | Volume per 10 Cycles | Storage |
|---------|---------------------|---------|
| ICB | 4.57 ml | 2°C to 8°C |
| LFN | 0.6 ml | -25°C to -15°C |
| EDP | 0.11 ml | -25°C to -15°C |

For more information on calculating volumes, see *Example Calculation* on page 29.

2   Transfer the measured volume of ICB to an empty 250 ml bottle.

3   Add the measured volume of LFN to the new bottle of ICB.

4   Rinse each tube of LFN with ICB to make sure that all LFN is transferred.

5   Add the measured volume of EDP to the ICB and LFN solution.

6   Rinse the tube of EDP with the ICB and LFN solution to make sure that all EDP is transferred.

7   Cap the bottle containing EDP, ICB, and LFN and invert to mix.

8   Set aside on ice.

## Load Paired-End Reagents

1   Make sure that the paired-end rack is not in use on the opposite flow cell for Read 2 resynthesis, Index 1 (i7) Read preparation, or Index 2 (i5) Read preparation.

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 reagent rack out of the reagent compartment using the rack handle.

4   Remove the cap from each reagent tube.

5   Place each tube onto the rack in the associated numbered positions.

| Position | Reagent | Description |
| --- | --- | --- |
| 10 | RMR | Resynthesis Mix |
| 11 | LMX2 | Linearization Mix 2 |
| 12 | BMX | Blocking Mix |
| 13 | AMX2 | Amplification Mix 2 |
| 14 | APM2 | AMX2 Premix |
| 15 | AT2 | 100% Formamide |
| 16 | HP7 or HP11 | Read 2 Sequencing Primer |
| 18 | HP3 | Denaturation Solution |
| 19 | HT2 | Wash Buffer |

6   Slide the reagent rack into the reagent compartment.Align the rack with the raised guide on the floor of the compartment.

7   Lower the sippers into the paired-end reagent tubes as follows.

a   Pull the handle towards you and lower it.

b   Inspect the sippers to make sure that they do not bend as they lower into the tubes.

c   Release the handle into the slot on the bottom end of the groove.

## Load ICB for Read 2

1   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.

2   Slide the reagent rack out of the reagent compartment.

3   Remove the ICB reagent bottle from position 1 and remove the funnel cap.

4   Place the funnel cap on the new bottle of ICB and load the bottle into position 1. Make sure that the conical end of the bottle rests in the indentation on the base of the rack.

5   Slide the reagent rack into the reagent compartment. Align the rack with the raised guide on the floor of the compartment.

6   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.

7   Close the reagent compartment door.

8    Select **Next** to resume 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 45*.

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.

# Chapter 5 Maintenance

## 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

A maintenance wash consists of washing the system with a maintenance wash solution prepared with Tween 20 and ProClin 300. Perform a maintenance wash when prompted by the software every 10 days or after a run, when a maintenance wash is an optional alternative to a water wash.

The Load Gasket screen opens with a maintenance wash every 10 days. Replace the 8-port gaskets in the front manifold and back manifold before proceeding to the wash, even if this screen does not appear.

### Prepare Maintenance Wash Solution

Prepare 5 liters of maintenance wash solutionfor 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 a run included an indexing read or paired-end turn, select **Yes** to wash PE reagent positions. Otherwise, select **No**.

3    Select **Next**.

4    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.

5    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.

6    Empty the waste bottle.

7    Select the **Wash solution loaded and template loading station closed** checkbox.

8    Select **Next**.

9    Remove the flow cell from the flow cell stage and set it aside.

10   Put on a new pair of powder-free latex gloves.

11   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 17**   Remove Used Manifold Gaskets
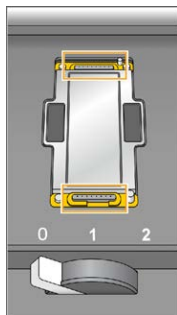


12   Place a new 8-port gasket in each slot on the front end and back end of the flow cell holder. Press lightly into position.

13  Reload the flow cell that you removed to install the new gaskets.

14  Make sure that the **Vacuum Engaged** checkbox is selected, and then select **Next**.

15  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.

16  Remove the waste tubes for the appropriate flow cell from the waste container.

17  Bundle the eight waste tubes with parafilm. Keep the tubes ends even.

18  Place the bundled tube ends into a 250 ml bottle.

19  Select **Next** to start the wash.

20  When the wash is complete, select **Return to Start**.

21  Measure the delivered volume.

| Positions | Delivered Volume |
| --- | --- |
| Eight SBS positions | 82 ml |
| 10 paired-end positions | 76 ml |
| All positions | 19.75 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.

22  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

## 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.<br>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.<br>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.<br>The vacuum did not seal.<br>Manifolds did not raise. | Remove the flow cell and repeat the cleaning steps.<br>Make sure that the gaskets are present and well-seated.<br>Reload the flow cell.<br>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.<br>Make sure that the gaskets are present and well-seated.<br>Reload the flow cell.<br>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*.<br>Look for white precipitate around the gaskets. If precipitate is present, replace the gaskets. Always replace gaskets before an instrument maintenance wash.<br>Confirm that the sipper assemblies are fully lowered and each sipper is in contact with the reagents. |

## 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.

## BaseSpace is Unavailable

If BaseSpace is not available, open Windows Services to confirm that the BaseSpace Broker has started. If it has not started, restart it. If services are running and BaseSpace is still unavailable, contact Illumina Technical Support.

## Stop and Resume a Run

Stopping a run might be necessary if the run was set up incorrectly, if the data quality is bad, or for a hardware error. When resuming a stopped run, select the appropriate normal stop option that allows the run to resume.

| Stop Option | Real-Time Analysis (RTA) Option | Able to Resume? |
|---|---|---|
| **Normal Stop (End of Lane\Chemistry)** | Keep As Is | Yes. The run resumes at the next chemistry or imaging command. |
| | Complete For Run | No. The run cannot be resumed. |
| | Complete For Read | Yes. The run resumes at the beginning of the next read. |
| **Normal Stop (End of Cycle)** | Keep As Is | Yes. The run resumes at the next cycle. |
| | Complete For Run | No. The run cannot be resumed. |
| | Complete For Read | Yes. The run resumes at the beginning of the next read. |
| **Immediate Stop** | No option | No. |

## Stop a Run

1 From the Run Overview screen, select **Stop** to open the Stop Menu.

**Figure 18** Stop Menu



Normal Stop (End of Lane\Chemistry)
Normal Stop (End of Cycle)
Immediate Stop
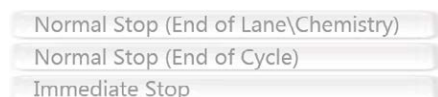
2 Select from the following stop options:
  ▶ **Normal Stop (End of Lane\Chemistry)**—Stops the run only after the current chemistry or imaging command is complete, and then places the flow cell in a safe state.

▶ **Normal Stop (End of Cycle)**—Stops the run after the current cycle is complete, and then places the flow cell in a safe state.

▶ **Immediate Stop**—Stops the run without completing the current operation and does *not* place the flow cell in safe state. You cannot resume a run that was stopped with this option.

3 Select from the following RTA options:

▶ **Keep As Is**—The run is stopped without any modifications to RTA. The run can resume where it was stopped.

▶ **Complete For Run**—RTA is stopped. The run info, run parameters, and recipe files are updated to reflect total cycles as the last cycle completed. Then RTA restarts to complete base calling for the run up to the point the run was stopped. The run cannot be resumed.

▶ **Complete For Read**—RTA is stopped. The run info, run parameters, and recipe files are updated to trim the length of the current read to the last cycle completed. Subsequent reads are not affected. Then RTA restarts to complete analysis for the current read. The run can resume at the beginning of the next read.

4 When the run is stopped, select **Return to Start** on the Run Overview screen.
The Welcome screen opens.

## Resume a Stopped Run

Use the following steps to resume a run that was stopped using a normal stop option with an RTA option that allows the run to be resumed.

> **NOTE**
>
> If the adjacent side is performing cluster generation or paired-end chemistry, the run resumes when the ongoing process is complete.

1 From the Welcome screen, select **Sequence**, and then select **Resume Run**.

2 From the Resume screen, select the appropriate run folder from the drop-down list.
The software resumes a run at the point where it was stopped, and defaults to the correct setup on the Resume screen.

3 Confirm the following default settings or select the appropriate point at which to resume the run. For more information, see *Example Settings for Resuming a Run* on page 51.

▶ **Resume At**—The read or point in the run to resume.

▶ **Start At Cycle**—The cycle to resume.

> **CAUTION**
>
> Do not select the point of paired-end turnaround, other than for Read 2 Primer rehybridization.

4 Select **Next** to proceed.
The software guides you through the remaining run setup steps.

## Example Settings for Resuming a Run

If the run was stopped after imaging lane 1 at cycle 23, the software automatically sets the resume run settings for Read 1 at cycle 23. The Resume screen displays these settings as follows.

▶ **Resume At:** Read 1

▶ **Start At Cycle:** 23

**Figure 19**  Example of Resuming at Cycle 23



Because the run in this example was stopped during an imaging step, **Imaging (no chemistry)** is selected automatically.

## 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.

> **CAUTION**
>
> Do not pause a run during imaging. Use the Normal stop, end of cycle, or end of lane feature to stop and resume a run.

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.

## Stagger Runs on Flow Cell A and Flow Cell B

When staggering runs on flow cell A and flow cell B, set up a new run and the software automatically pauses and resumes the run on the adjacent flow cell as needed. The system is placed in a safe state automatically.

1   Set up a new run.

> **NOTE**

> If the adjacent run is completing an imaging step, the software pauses the adjacent flow cell before you can load and prime reagents.

2   After loading the sequencing flow cell for the new run, close the compartment door.

3   Select **Start** to start the new sequencing run.

## Split SBS Kits

To perform a paired-end run that is shorter than 125 cycles per read, split a 250-cycle HiSeq v4 kit into 2 sets of reagents. One set provides reagents for a 126-cycle run with dual indexing and 1 prime. For a run that is shorter than 67 cycles, use a 50-cycle kit.

Splitting a 200-cycle TruSeq v3 kit provides enough reagents to perform up to 101 cycles with 1 prime. For a run that is shorter than 101 cycles, use a 50-cycle kit.

## Split a 250-Cycle Kit

1   Thaw reagents from the HiSeq v4 SBS kit (250 cycles). See *Thaw SBS Reagents* on page 12.

2   Label 7 250 ml bottles with the position number and reagent name, as follows.
   ▶   #1 IRM
   ▶   #3 USM
   ▶   #4 SB1
   ▶   #5 SB2
   ▶   #6 SB2
   ▶   #7 CRM
   ▶   #8 SB3

3   Add the following volume of each reagent into the appropriate 250 ml bottle to result in a second set of reagents. Prepare CRM last and then replace your gloves.

| Number | Reagent Name | Volume |
| --- | --- | --- |
| 1 | IRM | 72 ml |
| 3 | USM | 82 ml |
| 4 | SB1 | 100 ml |
| 5 | SB2 (for position #5) | 62 ml |
| 6 | SB2 (for position #6) | 62 ml |
| 7 | CRM | 72 ml |
| 8 | SB3 | 110 ml |

4   Use both sets of reagents for 2 concurrent runs or store 1 set at the following temperatures:
   ▶   Store IRM, USM, and CRM at -25°C to -15°C.
   ▶   Store SB1, SB2, and SB3 at 15°C to 30°C.

## Split a 200-Cycle Kit

1   Thaw reagents from the TruSeq v3 SBS kit (200 cycles). See *Thaw SBS Reagents* on page 27.

2   Label 7 250 ml bottles with the position number and reagent name, as follows.
   ▶   #1 ICB
   ▶   #3 SRE
   ▶   #4 SB1

- ▶ #5 SB2
- ▶ #6 SB2
- ▶ #7 CMR
- ▶ #8 SB3

3   Add the following volume of each reagent to the appropriate 250 ml bottle to result in a second set of reagents. Prepare CMR last and then replace your gloves.

| Number | Reagent Name | Volume |
| --- | --- | --- |
| 1 | ICB | 47 ml |
| 3 | SRE | 107 ml |
| 4 | SB1 | 100 ml |
| 5 | SB2 (for position #5) | 62 ml |
| 6 | SB2 (for position #6) | 62 ml |
| 7 | CMR | 107 ml |
| 8 | SB3 | 110 ml |

4   Use both sets of reagents for 2 concurrent runs or store 1 set at the following temperatures:
- ▶ Store CMR, SRE, and EDP at -25°C to -15°C.
- ▶ Store ICB, SB1, SB2, and SB3 at 2°C to 8°C.

## Prepare ICB

1   Add the contents of 2 tubes of LFN to 1 bottle of ICB.

2   Rinse each tube of LFN with ICB to make sure that all LFN is transferred.

3   Add 1.1 ml EDP to the ICB and LFN solution.

4   Return the unused portion of EDP to -25°C to -15°C storage.

5   Cap the bottle containing EDP, ICB, and LFN and invert to mix.

6   Set aside on ice.

## Primer Rehybridization

A rehybridization run repeats the sequencing primer hybridization step. If run metrics indicate low cluster numbers, low cluster intensities, or other concerns, perform primer rehybridization to rescue the flow cell. Primer rehybridization does not damage clusters.

## HiSeq v4 Flow Cell

All rehyb steps are performed on the HiSeq 2000. The kit includes primers for Read 1, Index 1 Read, Index 2 Read for single-read flow cells, and Read 2.

| Rehyb Kit Name | Workflow Instructions |
| --- | --- |
| HiSeq Multi-Primer Rehyb Kit v4 Catalog # GD-403-4001 | *HiSeq High Output Primer Rehybridization Guide (part # 15050105)* |

# TruSeq v3 Flow Cell

Read 1 primer rehyb is performed on the cBot. The rehyb kit includes a cBot reagent plate containing the Read 1 sequencing primer HP6. For Nextera libraries, use HP10 from the TruSeq Dual Index Sequencing Primer Box.

| Rehyb Kit Name | Workflow Instructions |
| --- | --- |
| TruSeq v2 cBot Multi-Primer Rehyb Kit Catalog # GD-304-2001 | *Read 1 Primer Rehyb on a TruSeq v3 or TruSeq v2 Flow Cell (part # 15018149)* |

# Appendix B Real-Time Analysis

## Real-Time Analysis Overview

Real-Time Analysis (RTA) runs on the instrument computer, performing base calling and assigning a quality score to each base call.

The software tracks the status of each tile and determines when to advance it to the next process step. When advancing a tile, RTA outputs a file for the completed step and then starts the next step. Thus, the software can determine the status of each tile based on which files exist. If RTA is terminated, it saves run data and can resume processing.

### Input Files

RTA requires the following input files:

▶ Cluster intensity files, which contain image analysis results.

▶ **RunInfo.xml**, which the control software generates automatically at the beginning of the run. From this file, RTA reads the run name, number of cycles, whether a read is indexed, and the number of tiles on the flow cell.

▶ **HiSeq.Configuration.xml**, which is an instrument configuration file in XML format.

▶ **RTA.exe.config**, which is a software configuration file in XML format.

RTA uses run parameters entered during run setup, and receives commands from the control software that include information about when to initiate and the location of **RunInfo.xml**.

### Output Files

Tiles are small imaging areas on the flow cell defined as 1 field of view by the camera. For each tile that is analyzed, RTA produces a set of quality-scored base call files and filter files as primary output. Other files support generation of primary output files.

▶ **Base call files**—For each tile that is analyzed, 1 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 1 filter (*.filter) file for each tile over the whole run. The filter file specifies whether clusters pass filter.

▶ **Cluster location files**—One cluster location (*.locs) file contains the X,Y coordinates for every cluster on the flow cell.

▶ **Statistics files**—For each cycle, 1 statistics file (*.stats) is produced. The statistics file contains aggregate statistics for the cycle.

Primary output files are used for subsequent data analysis. Use bcl2fastq for demultiplexing and conversion of .bcl files into FASTQ files, which can be used as input for alignment. To convert data from the HiSeq, use bcl2fastq 1.8.4, or later.

RTA provides real-time metrics of run quality stored as InterOp files. InterOp files are binary files containing tile, cycle, and read-level metrics, and are required for viewing metrics in Sequencing Analysis Viewer (SAV). For viewing metrics generated by RTA, use SAV v1.8.20 or later.

For details about each output file, see *Sequencing Output Files* on page 61.

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

## Error Handling

RTA stores log files in the RTALogs folder. If an error occurs, RTA creates an error log file called *Error.txt and records the error.

## Data Transfer

Throughout the run, RTA automatically copies data generated from raw image files to the output folder. If image analysis lags, RTA stops processing and places the flow cell in a safe state. Processing resumes when image data are available.

If RTA stops functioning, processing is automatically resumed during the next cycle at the appropriate point on the flow cell. Do not restart RTA manually.

Data transfer is complete when a marker file called Basecalling_Netcopy_complete.txt is generated. One of these files is generated for each read, and 1 is generated for the entire run.

## Real-Time Analysis Workflow

| | |
|---|---|
| Template generation | Maps cluster locations. |
| ↓ | |
| Registration and intensity extraction | Records the location of each cluster on the flow cell and determines an intensity value for each cluster. |
| ↓ | |
| Color matrix correction | Corrects cross talk between channels. |
| ↓ | |
| Empirical phasing correction | Corrects the effects of phasing and prephasing. |
| ↓ | |
| Base calling | Determines a base call for every cluster. |
| ↓ | |
| Quality scoring | Assigns a quality score to every base call. |

## Template Generation

Template generation locates and 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 random array of clusters on the flow cell, template generation requires image data from the first 5 cycles of the run. After the last template cycle for a tile is imaged, the template is generated.

Cluster positions are written to 1 cluster location (*.locs) file or compressed cluster location (*.clocs) file for each tile. For more information, see *Sequencing Output Files* on page 61.

## Registration and Intensity Extraction

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

▶ Registration aligns the images produced over every cycle subsequent to template generation against the template.

▶ 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, RTA 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, RTA 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 20** Phasing and Prephasing
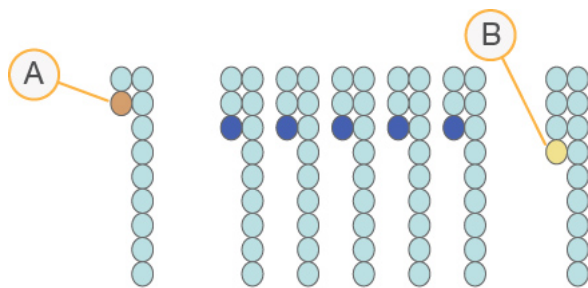


A    Read with a base that is phasing
B    Read with a base that is prephasing.

RTA 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 2000 using RTA begins after cycle 12.

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 the least reliable clusters from analysis results. Clusters pass filter when no more than 2 base calls have a chastity value below 0.6 in the first 25 cycles. Chastity is the ratio of the brightest base intensity divided by the sum of the brightest and the second brightest base intensities. Analysis reports represent the percentage of clusters passing filter as %PF.

Low quality clusters are removed from the raw cluster count during template generation, which yields a relatively high percentage of clusters passing filter.

# 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

RTA 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 2000. 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.

## Monitor Run Metrics

RTA automatically generates quality metrics when image analysis begins. However, not all metrics are available at the early cycles because some processes require multiple cycles to generate data.

| Data | Cycle |
|---|---|
| **Image analysis** | After cycle 5.<br>During the first 5 cycles of the run, RTA generates a template of cluster locations. |
| **Base calls** | After cycle 12.<br>Base calling begins after the color matrix is estimated at cycle 12. |
| **Phasing estimates** | After cycle 25.<br>The phasing corrections for the first 25 cycles determine the phasing estimate. |
| **Quality scores** | After cycle 25.<br>A quality score is generated for reads that pass the quality filter. Because quality scores require corrected intensities from future cycles, quality scoring always follows base calling. |

| Data | Cycle |
|------|-------|
| **Error rates** | After cycle 25.<br>Error rates are generated only when PhiX clusters are present and the Align to PhiX option is selected during run setup. |
| **Inline controls** | At cycle 52 of each read, or at the end of the run for runs with fewer than 52 cycles.<br>Inline controls are generated only for TruSeq library preparation methods.**\*** |
| **Index count** | After the index reads are complete.<br>The index count per lane is generated when a sample sheet is provided. |

\* Sequencing Analysis Viewer (SAV) v1.8.44 and later no longer includes the TruSeq Controls tab, where SAV reports results for the analysis of the inline controls.

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# Appendix C Output Files and Folders

## 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.<br>Data\Intensities\BaseCalls\L00[X]—Files are stored in per cycle folders for each lane.<br>**s_[Lane]_[Tile].bcl.gz**, where lane is the single-digit lane number and tile is the 4-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.<br>Data\Intensities |
| Filter files | The filter file specifies whether a cluster passed filters. Filter files are generated at cycle 26 using 25 cycles of data.<br>Data\Intensities\BaseCalls\L00[X]—Files are stored in 1 folder for each lane and tile.<br>**s_[lane]_[tile].filter** |
| InterOp files | Binary reporting files used for Sequencing Analysis Viewer. InterOp files are updated throughout the run.<br>**InterOp** folder |
| Log files | Record events and are updated throughout the run.<br>Data\RTALogs |
| Offsets files | Two offsets files are created for each run:<br>• **offsets.txt**—Contains tile offsets for every cycle and channel relative to the template.<br>• **SubTileOffsets.txt**—Contains the measured shift for each quadrant of each image relative to the frame of reference.<br>Data\Intensities\Offsets |
| Phasing files | Contains empirical phasing information by tile. Phasing files are created at the first cycle base called and updated after each cycle base called.<br>Data\Intensities\BaseCalls\Phasing<br>**EmpiricalPhasing_[lane]_[read]_[tile].txt**—Tile is represented with a 4-digit number that indicates surface, swath, and tile. |
| Real-Time Analysis configuration file | Created at the beginning of the run, the Real-Time Analysis configuration file lists settings for the run.<br>**Data\Intensities**<br>**RTAConfiguration.xml** |
| Statistics files | Statistics created at base calling for each cycle.<br>Data\Intensities\Basecalls\L00[X]\C[X.1]—Files are stored in 1 folder for each lane and 1 subfolder for each cycle. |
| Run information file | Lists the run name, number of cycles in each read, whether the read is an indexed read, and the number of swaths and tiles on the flow cell. The run info file is created at the beginning of the run.<br>**[Root folder]**<br>**RunInfo.xml** |
| Thumbnail files | A thumbnail image for each channel and tile in each swath at every cycle during imaging.<br>Thumbnail_Images\L00[X]\C[X.1]—Files are stored in 1 folder for each lane and 1 subfolder for each cycle.<br>**s_[lane]_[tile]_[channel].jpg**—Tile is represented with a 4-digit number that indicates surface, swath, and tile. See *Tile Numbering* on page 62. |

▶ 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
  ▶ 3 is for the third swath

▶ The last 2 digits represent the tile, 01 through 16. Tile numbering starts with 01 at the output end of the flow cell through 16 at the input end.

**Figure 21**  Tile Numbering
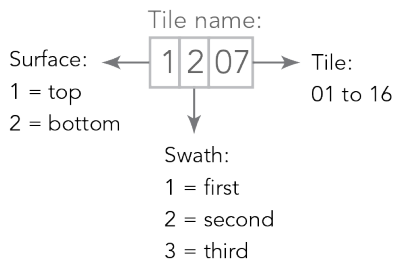


This example indicates a tile from the top surface of the flow cell, the second swath, and the seventh tile.

# Thumbnail Images

You can configure the control software to generate thumbnail images in *.jpg file format. Thumbnail images are generated for each cycle and base.

The control software collects images from 9 sections of a tile. The 9 images are combined into 1 thumbnail image and can be used to troubleshoot a run. Thumbnail images are not suitable for image analysis, but can be used for troubleshooting.

**Figure 22**  Thumbnail Image

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# Technical Assistance

For technical assistance, contact Illumina Technical Support.

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## Illumina Customer Support Telephone Numbers

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| Finland | +358 800918363 | +358 974790110 |
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**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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