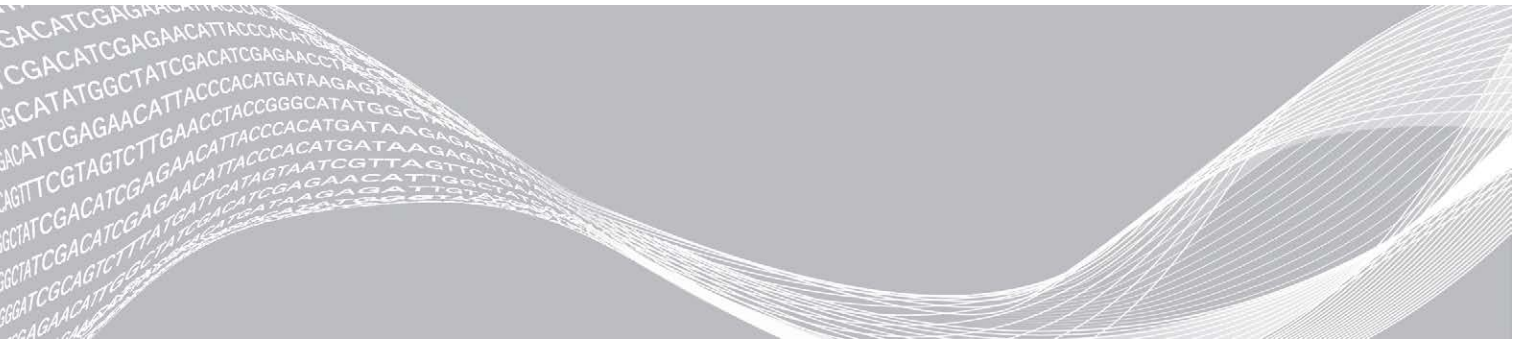
illumina®

NovaSeq 6000

Sequencing System Guide



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

Revision History

Document	Date	Description of Change
Material # 20018406 Document # 1000000019358 v01	March 2017	Corrected the name of a column on the Process Management screen to Sequencing.
Material # 20015871 Document # 1000000019358 v00	February 2017	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

The Illumina® NovaSeq™ 6000 Sequencing System packages scalable throughput and flexible sequencing technology into a production-scale platform with the efficiency and cost-effectiveness of a benchtop system.

Features

- **Scalable sequencing**–The NovaSeq 6000 scales up to production-level sequencing with high-quality data for a broad range of applications.
- **Adjustable output**–The NovaSeq 6000 is a dual flow cell system with a broad output range. Sequence 1 flow cell, or sequence 2 flow cells with different read lengths simultaneously. Mix and match flow cells and 3 different read lengths.
- **Patterned flow cell**–A patterned flow cell generates tightly spaced clusters. The reduced spacing between nanowells increases cluster density and data output.
- **On-instrument clustering**–The NovaSeq 6000 mixes the ExAmp reagents with library, amplifies the library on the surface of the flow cell, and performs cluster generation for a streamlined sequencing workflow.
- **High-throughput line scanning**–The NovaSeq 6000 uses 1 camera with bidirectional scanning technology to quickly image the flow cell in 2 color channels simultaneously.
- **Real-Time Analysis (RTA)**–The NovaSeq 6000 uses an implementation of RTA called RTA3. This integrated software analyzes images and calls bases.
- **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 to the environment in real time.
- **BaseSpace Clarity LIMS ready**–Improve operational efficiency with end-to-end tracking of samples and reagents, automated workflows, and integrated instrument operation.

Additional Resources

Visit the NovaSeq 6000 Sequencing System support pages on the Illumina website for software downloads, training resources, information about compatible Illumina products, and the following documentation.

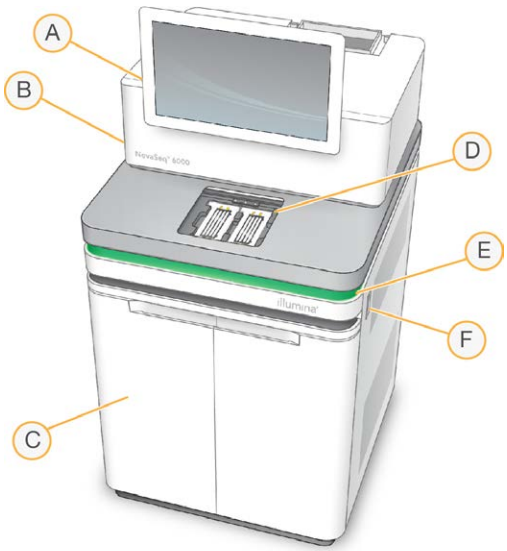
Resource	Description
Custom Protocol Selector	A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
NovaSeq Series Site Prep Guide (document # 1000000019360)	Provides specifications for laboratory space, electrical requirements, and environmental considerations.
NovaSeq Series Safety and Compliance Guide (document # 1000000019357)	Provides information about operational safety considerations, compliance statements, and instrument labeling.

Resource	Description
RFID Reader Compliance Guide (document # 1000000002699)	Provides information about the RFID reader in the instrument, including compliance certifications and safety considerations.
NovaSeq Series Custom Primers Guide (document # 1000000022266)	Provides information about replacing Illumina sequencing primers with custom sequencing primers.

Instrument Components

The NovaSeq 6000 Sequencing System comprises a touch screen monitor, a status bar, a power button with adjacent USB ports, and 3 compartments.

Figure 1 External Components



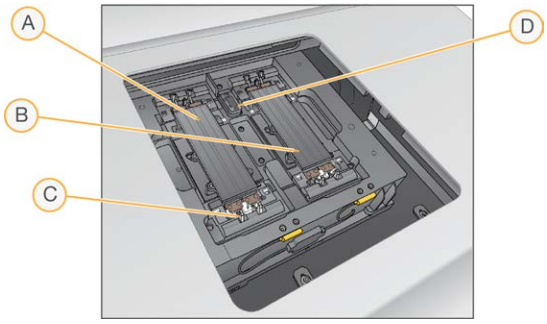
A **Touch screen monitor**–Displays the control software interface for instrument configuration and run setup.
B **Optics compartment**–Contains the optical components that enable dual surface imaging of flow cells.
C **Liquids compartment**–Contains reagents, buffer, and bottles for used reagents.
D **Flow cell compartment**–Holds the flow cells.
E **Status bar**–Indicates flow cell status as ready to sequence (green), processing (blue), or needs attention (orange).
F **Power and USB ports**–Provides access to the power button and USB connections for peripheral components.

Flow Cell Compartment

The flow cell compartment contains the flow cell stage, which holds flow cell A on the left and flow cell B on the right. Each side has 4 clamps that position and secure the flow cell.

The optical alignment target mounted on the flow cell stage diagnoses and corrects optical problems. When prompted by the control software, the optical alignment target realigns the system and adjusts camera focus to improve sequencing results.

Figure 2 Flow Cell Compartment



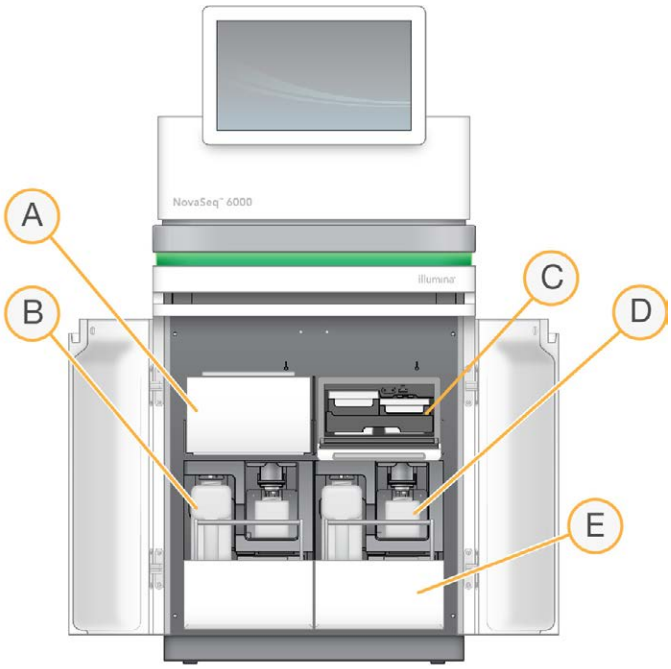
A Side A flow cell
B Side B flow cell
C Flow cell clamp (1 of 4 per side)
D Optical alignment target

The software controls the opening and closing of the flow cell compartment door. The door opens automatically to load a flow cell for a run or maintenance wash. After loading, the software closes the compartment door, moves the flow cell into position, and engages the clamps and vacuum seal. Sensors verify the presence and compatibility of the flow cell.

Liquids Compartment

Setting up a run requires accessing the liquids compartment to load reagents and buffer and empty used reagent bottles. French doors enclose the liquids compartment, which is divided into 2 matching sides for flow cell A and flow cell B.

Figure 3 Liquids Compartment Components



A **Reagent chiller**–Refrigerates the SBS and cluster cartridges.
B **Large used reagent bottle**–Holds used reagents from the SBS and buffer cartridges.
C **Reagent chiller drawer**–Color-coded positions hold the SBS cartridge on the left (gray label) and the cluster cartridge on the right (orange label).
D **Small used reagent bottle**–Holds used reagents from the cluster cartridge.
E **Buffer drawer**–Holds the large used reagent bottle on the left and the buffer cartridge on the right.

Used Reagent Bottles

The fluidics system is designed to route cluster cartridge reagents, which are potentially hazardous, to the small used reagent bottle. Reagents from the SBS and buffer cartridges are routed to the large used reagent bottle. However, cross-contamination between used reagent streams can occur. For safety, assume that both used reagent bottles contain potentially hazardous chemicals. The safety data sheet (SDS) provides detailed chemistry information.

NOTE

If the system is configured to collect used reagents externally, the stream to the large used reagent bottle is routed externally. Cluster cartridge reagents still go to the small used reagent bottle.

System Software

The instrument software suite includes integrated applications that perform sequencing runs, on-instrument analysis, and related functions.

- **NovaSeq Control Software (NVCS)**–Guides you through the steps to set up a sequencing run, controls instrument operations, and displays statistics as the run progresses. To demonstrate proper unloading and loading of consumables, NVCS plays instructional videos during run setup.

- **Real-Time Analysis (RTA)**–Performs image analysis and base calling during a run. NovaSeq 6000 uses RTA3, which has architecture, security, and other feature enhancements to optimize performance. For more information, see *Real-Time Analysis* on page 33.
- **Universal Copy Service**–Copies output files from RTA3 and NVCS to the output folder throughout a run. If applicable, the service also transfers data to BaseSpace Sequence Hub.

Status Icons

A status icon on the control software interface indicates run status. A number on the icon indicates the number of conditions for a status.

When a run status changes, the icon blinks to alert you. Select the icon to view a description of the condition. Select **Acknowledge** to clear the message, and then **Close** to close the dialog box.

Table 1 NVCS Status Icons

Status Icon	Status Name	Description
	Status okay	System is normal.
	Processing	System is processing.
	Warning	A warning has occurred and attention is required. Warnings do not stop a run or require action before proceeding.
	Error	An error has occurred. Errors require action before proceeding with the run.

Sequencing Consumables Overview

Each side of the NovaSeq 6000 requires a single-use NovaSeq 5000/6000 S2 Reagent Kit to perform a sequencing run. The kit includes a library tube, a flow cell, and the reagents required for a run. These components use radio-frequency identification (RFID) for accurate consumable tracking and compatibility.

Reagent Kits

Reagent kits for the NovaSeq 6000 system are available in the following configurations.

Kit Name	Illumina Catalog #
NovaSeq 5000/6000 S2 Reagent Kit (300 cycles)	20012860
NovaSeq 5000/6000 S2 Reagent Kit (200 cycles)	20012861
NovaSeq 5000/6000 S2 Reagent Kit (100 cycles)	20012862

Reagent Cartridges

Each of the 3 reagent cartridges is a single-use consumable with foil-sealed reservoirs prefilled with clustering, sequencing, and wash reagents. The cartridges load directly onto the instrument, and are color-coded and labeled to reduce loading errors. Guides in the reagent chiller drawer and buffer drawer ensure proper orientation.

SBS Cartridge

The NovaSeq 5000/6000 S2 SBS Cartridge is prefilled with sequencing reagents and has gray labeling. Each of the 3 reagent positions has an adjacent position reserved for the automatic post-run wash.

Figure 4 SBS Cartridge

Cluster Cartridge

The NovaSeq 5000/6000 S2 Cluster Cartridge is prefilled with clustering, indexing, and paired-end reagents, and includes a designated position for the library tube. Orange labeling distinguishes the cluster cartridge from the SBS cartridge.

Several reservoirs are reserved for the automatic post-run wash. The wash pumps 0.24% sodium hypochlorite (NaOCl) from position #17 and dilutes it to 0.12%. The 0.12% NaOCl is pumped to the ExAmp reagent and library positions, through the flow cell, and then to the used reagent bottles.

Figure 5 Cluster Cartridge

Removable Reservoir

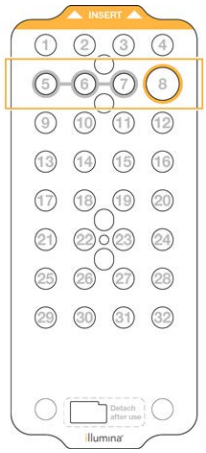
The cluster cartridge includes a denaturation reagent in position #30 that contains formamide, an organic amide and reproductive toxin. To facilitate safe disposal of any unused reagent after the sequencing run, this reservoir is removable.

Do not stack the SBS cartridge on top of the cluster cartridge, which can disengage position #30.

Reserved Reservoirs

The cluster cartridge has reserved reservoirs for custom primers and an empty position for the library tube. For sample traceability, the library tube is loaded in the cluster cartridge during run setup and remains with the cartridge through the end of the run.

Figure 6 Numbered Reservoirs



Position	Description
5, 6, and 7	Reserved for optional custom primers
8	Library tube

For more information on custom primers, see the *NovaSeq Series Custom Primers Guide (document # 1000000022266)*.

Buffer Cartridge

The NovaSeq 5000/6000 S2 Buffer Cartridge is prefilled with sequencing buffers and weighs up to 4.5 kg (10 lbs). A plastic handle facilitates carrying, loading, and unloading. Indentations on the top plate allow cartridges to be stacked.

Figure 7 Buffer Cartridge

Library Tube

The NovaSeq 5000/6000 Library Tube is a 16 mm tube that fits into position #8 of the cluster cartridge. Position #8 is labeled **Library Tube** and circled in orange for easy identification.

Pooled and denatured libraries are added to the library tube, which is then loaded uncapped into the cluster cartridge. The threaded cap allows for optional storage of libraries. After the run begins, libraries are mixed with ExAmp reagents in the library tube and then transferred automatically to both lanes of the flow cell.

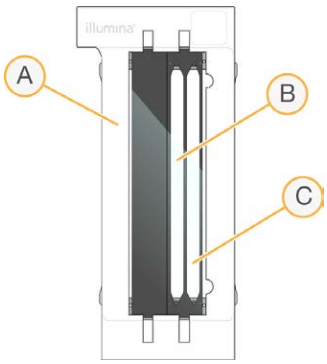
Figure 8 Library Tube

Flow Cell

The NovaSeq 5000/6000 S2 Flow Cell is a patterned flow cell encased in a cartridge. The flow cell is a glass-based substrate containing billions of nanowells in an ordered arrangement, which increases output reads and sequencing data. Clusters are generated in the nanowells from which the sequencing reaction is then performed.

The S2 flow cell has 2 lanes for sequencing a set of pooled libraries. Each lane is imaged in multiple swaths, and then the software divides the image of each swath into smaller portions called tiles. For more information, see *Flow Cell Tiles* on page 34.

Figure 9 Two-Lane Flow Cell



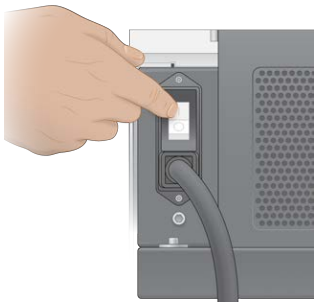
A Flow cell cartridge
B Lane 1
C Lane 2

Chapter 2 Getting Started

Start the Instrument

1 Switch the toggle power switch on the back of the instrument to the **|** (on) position.

Figure 10 Power Switch Location



2 Wait until the power button on the right side of the instrument glows blue, and then press it.

Figure 11 Power Button Location

3 When the operating system is loaded, log on to Windows using the user name and password for your site.

4 Open NovaSeq Control Software.
The software is launched and initializes the system. When initialization is complete, the Home screen appears.

Configure Settings

The control software includes settings to configure a manual or file-based run mode:

- **Manual**–The default mode that sends data to a specified output folder for later analysis.
- **File-Based**–An alternative mode that uses files from BaseSpace Clarity LIMS or other LIMS program to define run parameters.

When configuring the run mode, you must specify an existing location for the output folder or run setup folder. These folders are required, and an invalid location indicates that the specified location does not exist.

Both run modes include the option to send data to BaseSpace Sequence Hub for analysis.

Configure Manual Mode

1 From the Main Menu, select **Settings**.
The Settings screen opens to the Mode Selection tab.

2 Select **Manual**.

3 Enter or browse to a preferred network location for the output folder. Do not specify a location on the C:\ or D:\ drives because they do not have adequate space.
This setting is the default location. The output folder location can be changed on a per-run basis.

NOTE
If you are using BaseSpace Sequence Hub for run monitoring and storage, an output folder is optional.

4 [Optional] Clear the **Send Instrument Performance Data to Illumina** checkbox to prevent sending log files to Illumina.
When enabled, this option requires an external internet connection.

5 Select **Save**.

Configure File-Based Mode

1 From the Main Menu, select **Settings**.
The Settings screen opens to the Mode Selection tab.

2 Select **File-Based**.

3 Enter or browse to a preferred network location for the run setup folder.
The run setup folder contains LIMS files. During run setup, the software uses the library tube RFID to locate LIMS files for the current run.

4 [Optional] Enter or browse to a preferred network location for the output folder. Do not specify a location on the C:\ or D:\ drives because they do not have adequate space.
The output folder location can be changed on a per-run basis.

5 [Optional] Clear the **Send Instrument Performance Data to Illumina** checkbox to send log files to Illumina.
When enabled, this option requires an external internet connection.

6 Select **Save**.

Configure BaseSpace Sequence Hub

Use the following instructions to configure the default settings for BaseSpace Sequence Hub. During run setup, you can disable BaseSpace Sequence Hub for the current run and change settings for run monitoring and storage. Connecting to BaseSpace Sequence Hub requires an internet connection.

1 From the Main Menu, select **Settings**.
The Settings screen opens to the Mode Selection tab.

2 Select the **BaseSpace Sequence Hub** checkbox.

3 Select a Configuration option:
- **Run Monitoring and Storage**–Sends run data to BaseSpace Sequence Hub for remote monitoring and data analysis. This option requires a sample sheet.

- **Run Monitoring Only**–Sends InterOp, log, and other non-CBCL run files to BaseSpace Sequence Hub so runs can be monitored remotely.

4 From the Hosting Location drop-down menu, select **EU (Frankfurt)** or **USA (N. Virginia)**.
This option determines where data are uploaded to.

5 If you are a BaseSpace Enterprise subscriber:

a Select the Private Domain checkbox.

b Enter the domain named used for single sign-on to BaseSpace Sequence Hub.

6 Select **Save**.

Manage Disk Space

Use the Process Management screen to monitor run progress, delete runs, and otherwise manage disk space. The screen provides access to the Compute Engine (CE) and hard drive (C:\). Never delete files and folders from C:\ directly.

Process Management displays available disk space, space used on CE and C:\, and the status of runs using disk space. Run Date and Name columns identify each run. Sequencing, Network, and BaseSpace columns show the file copy status of each process for a run.

Before a dual flow cell run can begin, CE requires at least 5.4 GB of space per cycle and the hard drive requires 10 GB of space.

Delete a Run

After data transfer is complete, delete the current run to clear space for a subsequent run. Deleting the run clears CE and C:\ without removing system maintenance files or affecting the network and BaseSpace Sequence Hub copying. Runs that are sequencing cannot be deleted.

1 From the Main Menu, select **Process Management**.

2 [Optional] Make sure that each process for the run displays a green checkmark, which indicates that data transfer is complete.
You can delete a run that has not completed transfer to a network or BaseSpace Sequence Hub, but all run data are lost.

3 Select **Delete Run**, and then select **Yes** to confirm.

4 Select **Done**.

User-Supplied Consumables and Equipment

The following consumables and equipment are used for sequencing and system maintenance.

User-Supplied Consumables

Consumable	Supplier	Purpose
1 N NaOH (sodium hydroxide)	General lab supplier	Diluting to 0.2 N for denaturing libraries.
10 mM Tris-HCl, pH 8.5	General lab supplier	Diluting libraries before denaturation.

Consumable	Supplier	Purpose
400 mM Tris-HCl, pH 8.0	General lab supplier	Neutralizing libraries and an optional PhiX control after denaturation.
Centrifuge bottle, 500 ml	General lab supplier	Diluting Tween 20 for a maintenance wash.
Centrifuge tube, 30 ml	General lab supplier	Diluting NaOCl for a maintenance wash.
Isopropyl alcohol wipes, 70% or Ethanol, 70%	VWR, catalog # 95041-714, or equivalent General lab supplier	Cleaning components before a run and general purpose.
Disposable gloves, powder-free	General lab supplier	General purpose.
Lab tissue, low-lint	VWR, catalog # 21905-026, or equivalent	Drying the flow cell stage and general purpose.
Microcentrifuge tube, 1.5 ml	VWR, catalog # 20170-038, or equivalent	Combining volumes when diluting NaOH and library.
NaOCl, 5%	Sigma-Aldrich, catalog # 239305	Performing a maintenance wash.
Tween 20	Sigma-Aldrich, catalog # P7949	Performing a maintenance wash.
Water, laboratory-grade	General lab supplier	Diluting NaOH for denaturing libraries. Diluting Tween 20 and sodium hypochlorite for a maintenance wash.
[Optional] PhiX Control v3	Illumina, catalog # FC-110-3001	Spiking in a 1% PhiX control.

Guidelines for Laboratory-Grade Water

Always use laboratory-grade water or deionized water to perform instrument procedures. Never use tap water. Use only the following grades of water or equivalents:

- Deionized water
- Illumina PW1
- 18 Megohms (MΩ) water
- Milli-Q water
- Super-Q water
- Molecular biology grade water

User-Supplied Equipment

Item	Source
Freezer, -25°C to -15°C, frost-free	General lab supplier
Ice bucket	General lab supplier
Pipette, single channel, 20 µl	General lab supplier
Pipette, single channel, 200 µl	General lab supplier
Pipette, single channel, 1000 µl	General lab supplier
Refrigerator, 2°C to 8°C	General lab supplier
Tub, water baths*	General lab supplier

* Use a tub that can accommodate 2 reagent cartridges and the appropriate water level. For example, 61 cm (24 in) × 91.4 cm (36 in) × 25.4 cm (10 in).

Chapter 3 Sequencing

Methods

This chapter describes the sequencing protocol for the NovaSeq 6000 Sequencing System. For a successful run:

- Make sure that you have the required equipment and consumables.
- Follow the protocols in the order shown, using the specified volumes, temperatures, and durations.
- Unless a stopping point is specified in the protocol, proceed immediately to the next step.

Sequencing Overview

Sequencing on the NovaSeq 6000 comprises the following steps.

Cluster Generation

In preparation for cluster generation, 525 µl ExAmp Master Mix is automatically mixed with 225 µl library onboard the instrument, yielding a final concentration of 200-500 pM. Single DNA molecules are then bound to the surface of the flow cell and then simultaneously amplified to form clusters.

Sequencing

Clusters are imaged using bidirectional scanning and 2-channel sequencing chemistry. The camera uses red and green sensors to image each swath and simultaneously generate red and green images of the whole swath. After imaging, the 2 images are divided into red and green tiles that RTA3 uses for base calling, filtering, and quality scoring. This process is repeated for each cycle of sequencing.

Analysis

As the run progresses, the control software automatically transfers base call (*.cbcl) files to the specified output folder location for data analysis.

Several analysis methods are available and depend on your application. For more information, visit the BaseSpace Sequence Hub support page on the Illumina website.

Staggering Runs

You can set up a dual or single flow cell run, or stagger 2 single flow cell runs. For instructions, see *Stagger Runs on Flow Cell A and Flow Cell B* on page 28.

Number of Cycles in a Read

In a sequencing run, the number of cycles performed in Read 1 and Read 2 is 1 more cycle than the number of cycles analyzed. For example, to perform a paired-end 150-cycle run, enter 151 in the Read 1 and Read 2 fields for a total of 302 cycles (2 × 151). At the end of the run, 2 × 150 cycles are analyzed. The extra cycle in each read is used for phasing and prephasing calculation.

Sequencing Workflow

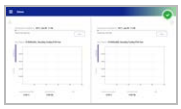
Prepare new reagent cartridges by thawing and inspecting.

Pool and denature libraries, and then load the library tube into the thawed cluster cartridge.
Prepare a new flow cell by bringing it to room temperature for 30 minutes and then removing the packaging.

From the software interface, select **Sequence** and specify a dual or single flow cell run.

Unload consumables from the previous run and load consumables for the current run.
Empty and reload the used reagent bottles.

Specify run parameters from the Run Setup screen. If BaseSpace Sequence Hub is configured, sign in from the Log In screen.
After pre-run checks, the run starts automatically.



Monitor the run from the Sequence screen, BaseSpace Sequence Hub if run monitoring is enabled, or a network computer using Sequencing Analysis Viewer.
Data are transferred to the specified output folder.

An instrument wash begins automatically when sequencing is complete.

Prepare the SBS and Cluster Cartridges

Thaw the SBS and cluster cartridges using 1 of 2 methods: a 3-hour thaw in a water bath or an overnight thaw in a refrigerator with subsequent room-temperature thawing. If you must refreeze reagents, do so immediately after thawing.

Wire thaw racks provided with the instrument prevent the cartridges from capsizing in water baths.

Figure 12 Reagent Cartridges in Wire Thaw Racks

The buffer cartridge is used directly from 19°C to 25°C storage, and does not require preparation.

1 Remove the SBS and cluster cartridges from -25°C to -15°C storage.

2 To thaw reagents using the 3-hour thaw method:

   a Place each cartridge into a wire thaw rack.
   b Thaw in a room temperature water bath (19°C to 25°C) for 3 hours. Submerge about halfway.
   c Dry the cartridge bases using paper towels.

3 To thaw reagents using the overnight method:

   a Thaw both cartridges in a refrigerator (2°C to 8°C) for at least 14 hours.
   b Thaw the cluster cartridge at room temperature for 2 hours.
   c Place the SBS cartridge into a wire thaw rack and thaw in a room temperature water bath (19°C to 25°C) for 2 hours. Submerge about halfway.
   d Dry the SBS cartridge base using paper towels.

4 Invert each cartridge 10 times to mix reagents.

5 Inspect the underside of each cartridge to make sure that reagents are thawed. The reservoirs must be free of ice.

6 Inspect the foil seals for water. If water is present, blot dry with a lint-free tissue.

7 Gently tap the bottom of each cartridge on the bench to reduce air bubbles.

8 Set aside at room temperature for up to 4 hours. Alternatively, store at 2°C to 8°C for up to 24 hours when you cannot load reagents onto the instrument within 4 hours.

Denature Libraries and Add PhiX

Use the following instructions to denature libraries and an optional PhiX control for a sequencing run. Make sure that volumes are measured and dispensed accurately. The system transfers reagents to the library tube for onboard mixing before sequencing starts, so an incorrect volume of library can cause errors or failures during clustering.

The instructions apply to supported Illumina libraries and assume an insert size typical for the associated library type. Dilute to a loading concentration appropriate for the library type. A loading concentration that is too low or too high negatively impacts the percentage of clusters passing filter (%PF).

Achieving quality data requires accurate library quantification to optimize the library loading concentration. For recommended quantification methods, see the documentation for your library prep kit.

Prepare a Fresh Dilution of NaOH

A fresh dilution of 0.2 N NaOH denatures libraries for cluster generation. To prevent small pipetting errors from affecting the final NaOH concentration, prepare at least 50 µl of freshly diluted NaOH.

1 Combine the following volumes in a microcentrifuge tube:
- Laboratory-grade water (40 µl)
- Stock 1 N NaOH (10 µl)

The total volume is 50 µl 0.2 N NaOH.

2 Invert several times to mix or vortex thoroughly. Use within ***12 hours***.

Create a Normalized Library Pool

Use the following instructions to normalize libraries to at least 1 nM and then pool. Libraries loaded onto an S2 flow cell must be combined into 1 normalized pool with a total volume of 150 µl.

For best results, pool and denature libraries for immediate sequencing.

Create a Set of Normalized Libraries

1 Determine the final pool concentration based on the final loading concentration:

Final Concentration (pM)	Pooled Library Loading Concentration (nM)	Total Volume (µl)
200	1	150
250	1.25	150
300	1.5	150
350	1.75	150
400	2	150
450	2.25	150
500	2.5	150

2 Normalize your libraries to the desired pooled library loading concentration using 10 mM Tris-HCl pH, 8.5. For assistance diluting libraries to the appropriate concentration, see the Pooling Calculator at support.illumina.com/help/pooling-calculator/pooling-calculator.html.

Pool Libraries and Add an Optional PhiX Control

1 Combine the appropriate volume of each normalized ≥ 1 nM library in a new microcentrifuge tube to result in a total pool volume of 150 µl.

2 [Optional] Store remaining ***unpooled*** ≥ 1 nM libraries at -25°C to -15°C.

3 [Optional] Spike-in 1% nondenatured PhiX to the tube of 150 µl nondenatured library pool.
For example, add 1.5 µl 2.5 nM PhiX to a 150 µl 2.5 nM library pool. The PhiX concentration must match the pooled library loading concentration. The PhiX volume is always 1.5 µl.

NOTE

When spiking in PhiX, 1% is the recommended amount for well-balanced libraries. Low-diversity libraries can require more. To use a PhiX control with low-diversity libraries, contact Illumina Technical Support for guidance.

Denature Library Pool and Optional PhiX Control

1 Add 37 µl 0.2 N NaOH to the tube of nondenatured library pool and optional PhiX.
The resulting volume is 187 µl, or 188.5 µl with PhiX.

2 Cap and then vortex briefly.

3 Centrifuge at 280 × g for 1 minute.

4 Incubate at room temperature for 8 minutes.

5 Add 38 µl 400 mM Tris-HCl, pH 8.0.
The resulting volume is 225 µl, or 226.5 µl with PhiX.

6 Cap and then vortex briefly.

7 Centrifuge at 280 × g for 1 minute.

8 Transfer 225 µl denatured library mixture to the library tube provided with the kit.

CAUTION

Immediately proceed to loading the library tube and setting up the run. Load the reagent cartridges onto the instrument within ***30 minutes***.

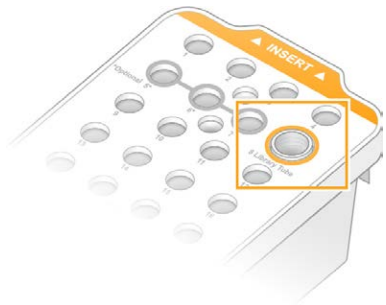
9 [Optional] If you cannot start the run immediately, cap the library tube and choose a storage option:
- Store at 2°C to 8°C for up to 1 week.
- Store at -25°C to -15°C for up to 3 weeks. After thawing, do not refreeze.

Store only if necessary. Storage can increase duplicates, which decrease yield.

Load Library Tube

1 Without disturbing the library at the bottom of the tube, insert the uncapped library tube into the **Library Tube** position (#8) of the cluster cartridge.

Figure 13 Uncapped Library Tube Loaded Into Position #8



Prepare the Flow Cell

1 Remove a new flow cell package from 2°C to 8°C storage.

2 Put on a new pair of powder-free gloves to avoid contaminating the glass surface of the flow cell.

3 With the package over a flat surface, peel open the foil package from the end with the angled seal.

4 Remove the clamshell case from the foil package.

5 Open the clamshell case and remove the flow cell. Grasp the flow cell by the sides to avoid touching the glass or gaskets on the bottom of the cartridge.

6 Clean the glass surface of the flow cell with a lint-free alcohol wipe. Dry with a low-lint lab tissue.

Set Up a Sequencing Run

1 Remove any items from the surface of the instrument.
Keep the surface clear during the sequencing run and avoid leaning on the instrument. Pressure to the flow cell door can cause it to open, which stops the run.

2 From the Home screen, select **Sequence**, and then select a single or dual flow cell run:
- **A+B**–Set up a dual flow cell run.
- **A**–Set up a single flow cell run on side A.
- **B**–Set up a single flow cell run on side B.

The software initiates the series of run setup screens, starting with Load.

3 Select **OK** to acknowledge the warning and open the flow cell door.

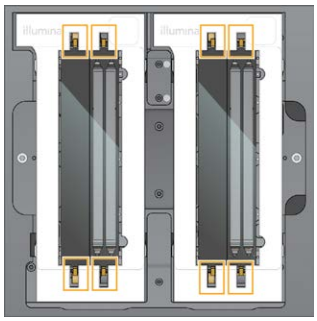
Load the Flow Cell

1 If present, remove the flow cell from the previous run.

2 Clean the flow cell holder and stage and the glass surface of the optical alignment target with an alcohol wipe. Dry with a lint-free tissue to remove alcohol residue.

3 Align the flow cell over the 4 raised clamps and place it on the flow cell stage.

Figure 14 Loaded Flow Cells Aligned Over Clamps



4 Select **Close Flow Cell Door**.
The flow cell door closes, the sensors and RFID are checked, and the flow cell ID appears on the screen.

Load the SBS and Cluster Cartridges

1 Open the French doors, and then open the reagent chiller door.

2 Remove the used SBS and cluster cartridges
The used cartridges have pierced foil seals.

3 Dispose of unused contents in accordance with applicable standards.

For safe disposal of position #30 of the cluster cartridge, see *Detach Position #30* on page 23.

4 Load the prepared cartridges into the reagent chiller drawer so that the **Insert** labels face the back of the instrument:
- Place the SBS cartridge (gray label) into the left position.
- Place the cluster cartridge (orange label) containing the uncapped library tube into the right position.

Figure 15 Loaded Reagent Cartridges

5 Slide the drawer into the chiller, and then close the reagent chiller door.
The sensors and RFIDs are checked. The IDs for the library tube and the 2 cartridges appear on the screen.

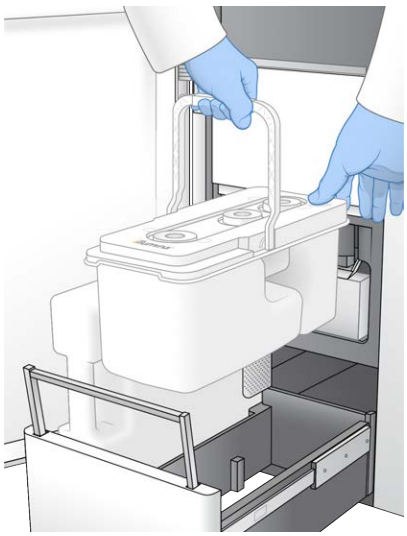
Load the Buffer Cartridge

1 Pull the metal handle to open the buffer drawer.

2 Remove the used buffer cartridge from the right side of the buffer drawer.
The used buffer cartridge has pierced foil seals.

3 Place a new buffer cartridge into the buffer drawer so that the **Illumina** label faces the front of the instrument. Align the cartridge with the raised guides on the drawer floor and sides.
When properly loaded, the buffer cartridge is evenly seated and the drawer can close.

Figure 16 Load the Buffer Cartridge



Empty Used Reagent Bottles

Use the following instructions to empty the used reagent bottles with ***every*** sequencing run. Even if your system is configured to route used reagents externally, the small bottle collects used reagents and the large bottle must be in place.

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.

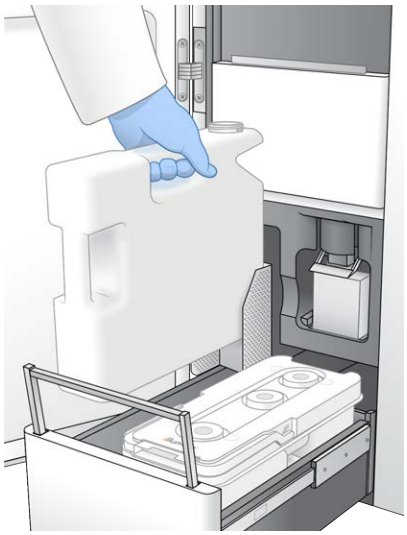
1 Remove and empty the small used reagent bottle as follows.

   a Raise the lever and remove the small used reagent bottle from the alcove.
   b Seal the bottle opening with the threaded cap to prevent spills.
   c Keeping the contents separate from the contents of the other bottle, discard in accordance with applicable standards.
   d Return the uncapped bottle to the alcove, and lower the lever.

2 Remove and empty the large used reagent bottle as follows.

   a Using the top handle, remove the large used reagent bottle from the left side of the buffer drawer.
   b Seal the bottle opening with the threaded cap to prevent spills.
   c Discard the contents in accordance with applicable standards. Grip both handles when emptying.
   d Return the uncapped bottle to the buffer drawer.

Figure 17 Returning the Empty Bottle



3 Close the buffer drawer, and then close the French doors.
The sensors and RFID are checked, and the buffer cartridge ID appears on the screen.

4 Select the checkbox acknowledging that both used reagent bottles are empty.

WARNING

Failure to empty the used reagent bottles can result in a terminated run and overflow, which damages the instrument and poses a safety risk.

5 Select the available button:
- **Log In**–Opens the Log In screen. Proceed to *Sign In to BaseSpace Sequence Hub*.
- **Run Setup**–Skips BaseSpace Sequence Hub sign-in and opens the Run Setup screen. Proceed to *Enter Run Parameters* on page 21.

Which button is available depends on whether the system is configured for BaseSpace Sequence Hub.

Sign In to BaseSpace Sequence Hub

1 [Optional] Update BaseSpace Sequence Hub settings for the current run:
- To disable BaseSpace Sequence Hub, clear the **BaseSpace Sequence Hub** checkbox and then select **Run Setup** to proceed without signing in.
- To send run data to BaseSpace Sequence Hub for remote monitoring and data analysis, select **Run Monitoring and Storage**. This option requires a sample sheet.
- To send only InterOp files to BaseSpace Sequence Hub to monitor the run remotely, select **Run Monitoring Only**.

2 Enter your BaseSpace Sequence Hub username and password, and then select **Sign In**.

3 If prompted, select a workgroup to upload run data to, and then select **Run Setup**.
You are prompted only if you belong to multiple workgroups.

Enter Run Parameters

1 In the Run Name field, enter a name of your preference to identify the current run.

2 Enter the number of cycles for each read in the sequencing run.
- **Read 1**–Enter a value up to 151 cycles.
- **Index 1**–Enter a value up to 20 cycles for the Index 1 (i7) primer.
- **Index 2**–Enter a value up to 20 cycles for the Index 2 (i5) primer. For a single-index run, enter **0**.
- **Read 2**–Enter a value up to 151 cycles. This value is typically the same as the Read 1 value. For a single-read run, enter **0**.

3 Expand **Advanced Options** to apply settings for the current run. These settings are optional unless otherwise indicated.
- **Custom Primers**–Select the **Custom Primers** checkbox, and then select the appropriate checkboxes:
  - **Read 1**–Use custom primer for Read 1.
  - **Read 2**–Use custom primer for Read 2.
  - **Custom Index**–Use custom primer for Index 1.
- **Output Folder**–Select **Browse** to change the output folder for the current run. An output folder is required when the run is not connected to BaseSpace Sequence Hub for storage.
- **Attachment**–Select **Browse** to upload a sample sheet, which is required when using BaseSpace Sequence Hub for run monitoring and storage, or other CSV file. The attachment is copied to the output folder and does not affect run parameters.

4 Select **Review**.
The software confirms that the specified parameters are appropriate for the recipe.

Confirm Run Parameters

1 Confirm the run parameters displayed on the Review screen.

2 [Optional] Select **Back** to return to the Run Setup screen and edit run parameters.

3 Select **Start Run**.
The pre-run checks are started automatically.

Review Pre-Run Checks

The sequencing run starts automatically after a successful pre-run check, which takes about 5 minutes. Any errors require resolution before the run can start. See *Pre-Run Check Errors* on page 29.

NOTE
To avoid overfilling the hard drive, do not copy any data to C:\ after the run starts.

1 If pre-run checks fail, select **Retry** to restart the failed check or **Retry All** to restart all checks. Select the **Error** icon to see error details.

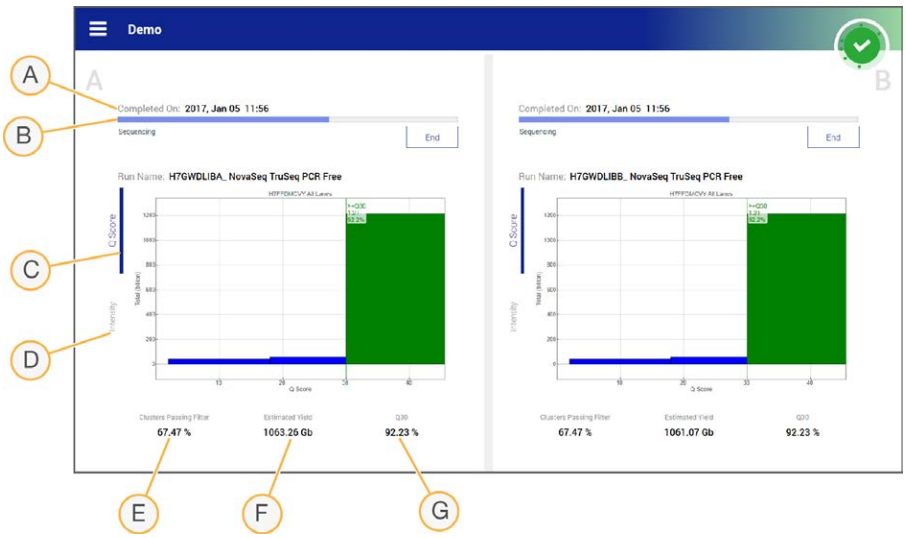
2 If the alignment check fails, resolve the error as follows.

a Select **Reload**, and then select **OK** to confirm returning to the Load screen.
b Remove any items from the top of the instrument, and then select **OK**.
c Reload the flow cell, and then select **Run Setup**.
d Proceed through each screen to reread each RFID and return to the Pre-Run Checks screen.
e Redo the check.

Monitor Run Progress

1 Monitor run progress, intensities, and quality scores as metrics appear on the screen.
For more information on run metrics, see *Real-Time Analysis* on page 33.

Figure 18 Sequencing Run Progress and Metrics



A **Time to completion**–The run completion date and time (yyyy-mm-dd hh:mm).
B **Run progress**–The current run step. The progress bar is not proportional to the run rate of each step.
C **Q-scores**–The distribution of quality scores (Q-scores).
D **Intensity**–The value of cluster intensities of the 90th percentile for each tile. Plot colors indicate each base: red is A, green is C, blue is G, and black is T.
E **Clusters passing filter (%)**–The percentage of clusters passing filter.
F **Estimated yield (Gb)**–The number of bases projected for the run.
G **Q30**–The percentage of base calls for the run that have a Q-score of ≥ 30.

Run Metrics

The software displays metrics generated during the run. Metrics appear in the form of plots, graphs, and tables based on data generated by RTA3 and written to InterOp files.

Clustering takes about 2 hours, then sequencing begins with cycle 1. Metrics are updated as sequencing progresses. Clusters passing filter, yield, and quality scores are available after cycle 26.

Processing Status

The Process Management screen lists the status of each run. From the Main Menu, select **Process Management**.

For each run name, Process Management lists the status of the following processes:

- **Sequencing**–Based on the processing of CBCL files.
- **Network**–Based on file transfer using Universal Copy Service.
- **BaseSpace**–Based on file upload to BaseSpace Sequence Hub, if applicable.

When a process is complete, a green checkmark appears. A red exclamation mark indicates a problem.

Data Transfer

When data transfer is complete, RTAComplete.txt, CopyComplete.txt, and SequenceComplete.txt appear in the output folder. To confirm successful data transfer, make sure that CopyComplete.txt is present and the Process Management screen displays green checkmarks for all processes.

Detach Position #30

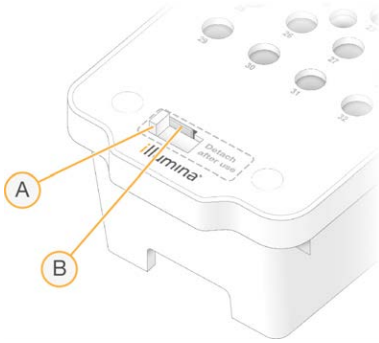
The reservoir in position #30 of the cluster cartridge contains formamide. It is removed from the used cluster cartridge and discarded separately.

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.

1 Wearing gloves, press the white plastic tab labeled **Detach after use** to the right.
2 With a hand or surface under the reservoir, press the clear plastic tab toward the Illumina label to release the reservoir from under the cluster cartridge.

Figure 19 Removable Position #30



A White plastic tab to detach
B Clear plastic tab to release

3 Dispose of the reservoir in accordance with applicable standards.

Automatic Post-Run Wash

When sequencing is complete, the software initiates an automatic post-run wash that takes about 80 minutes. The system dilutes 0.24% NaOCl from the cluster cartridge to 0.12% to flush template from the ExAmp reagent positions and prevent cross-contamination.

When the wash is complete, the system is placed in a safe state and the Home button becomes active. Leave consumables in place until the next run. After the wash, the sippers remain in the SBS and cluster cartridges to prevent air from entering the system. The sippers in the buffer cartridge are raised so the used reagent bottles can be emptied.

Chapter 4 Maintenance

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

The maintenance wash flushes the system with a user-supplied dilution of Tween 20 and a user-supplied dilution of NaOCl. Wash duration is about 80 minutes. The wash requires a used buffer cartridge and the SBS wash cartridge, cluster wash cartridge, and 4-lane wash flow cell provided with the instrument.

Like the reagent cartridges, the wash cartridges are color-coded to prevent loading errors. The SBS wash cartridge has a center well for the Tween 20 dilution. The NaOCl dilution is added to a reservoir on the cluster wash cartridge. The dilutions are pumped from the wash cartridges to the flow cell, used reagent, and every cartridge reservoir to wash all sippers.

Figure 20 SBS Wash Cartridge (Left) and Cluster Wash Cartridge (Right)

If the system is idle for 14 days, an automatic post-run wash was unsuccessful, or a run was ended without a wash, the software prompts for a maintenance wash. Systems that successfully complete runs and post-run washes within a 14-day period do not require a maintenance wash.

Prepare Dilutions of Tween 20 and NaOCl

1 Add 400 ml laboratory-grade water to a 500 ml centrifuge bottle.

2 Add 0.2 ml 100% Tween 20 to result in at least 400 ml 0.05% Tween 20 wash solution.
Using a freshly prepared dilution of Tween 20 prevents the introduction of microbes into the fluidics system.

3 Invert to mix.

4 Remove the lid from the center well of the SBS wash cartridge.

5 Add 400 ml wash solution to the center well. Fill to at least the fill line, which indicates the minimum required volume.
The other reservoirs remain empty.

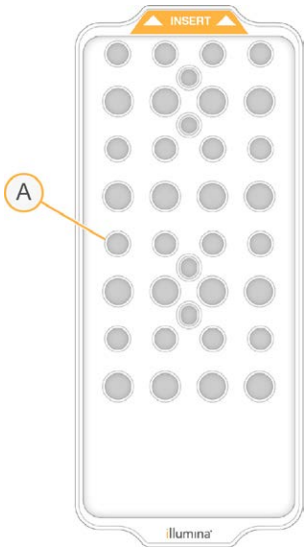
Figure 21 Center Well Filled to 400 ml

6 Combine the following volumes in a 30 ml centrifuge tube to prepare 20 ml of 0.25% NaOCl:
- 5% NaOCl (1 ml)
- Deionized water (19 ml)

7 Invert to mix.

8 Add 5 ml 0.25% NaOCl to the cluster wash cartridge.
The correct reservoir is equivalent to position **#17** of the prefilled reagent cartridge. All other reservoirs remain empty.

Figure 22 Position for 0.25% NaOCl



Load the Wash Flow Cell

1 Remove any items from the surface of the instrument.
Keep the surface clear during the maintenance wash and avoid leaning on the instrument. Pressure to the flow cell door can cause it to open, which stops the wash.

2 From the Home screen, select **Wash**, and then select which side to wash:
- **A+B**–Wash both sides simultaneously.

- **A**–Wash side A only.
- **B**–Wash side B only.

The software initiates the series of wash screens.

3 Select **OK** to acknowledge the warning and open the flow cell door.

4 If a wash flow cell is not already present, load one.

5 Select **Close Flow Cell Door**.
The door closes, the sensors and RFID are checked, and the flow cell ID appears on the screen.

Load the Wash Cartridges

1 Open the French doors, and then open the reagent chiller door.

2 Remove the used SBS and cluster reagent cartridges. Dispose of unused contents in accordance with applicable standards.
For safe disposal of position #30 of the cluster cartridge, see *Detach Position #30* on page 23.

3 Load the wash cartridges into the reagent chiller drawer so that the **Insert** labels face the back of the instrument:
- Place the SBS cartridge (gray label) into the left position.
- Place the cluster cartridge (orange) label into the right position.

4 Slide the drawer into the chiller, and then close the reagent chiller door.
The sensors are checked and the RFID for each cartridge is scanned and displayed on the screen.

5 Open the buffer drawer.

6 If not already present, load a used buffer cartridge.

Empty Used Reagent Bottles

Use the following instructions to empty the used reagent bottles with ***every*** maintenance wash. Even if your system is configured to route used reagents externally, the small bottle collects used reagents and the large bottle must be in place.

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.

1 Remove the small used reagent bottle and discard the contents in accordance with applicable standards. Keep the contents separate from the contents of the other bottle.

2 Return the small used reagent container to the alcove.

3 Remove the large used reagent bottle and discard the contents in accordance with applicable standards.

4 Return the large used reagent bottle to the buffer drawer.

5 Close the buffer drawer, and then close the French doors.
The sensors and RFIDs are checked. The ID of each wash component appears on the screen.

Start the Wash

1 Select the checkbox acknowledging that both used reagent bottles are empty, and then select **Start Wash**. The wash starts and the estimated time of wash completion is displayed.

WARNING

Failure to empty the used reagent bottles can result in a terminated wash and overflow, which damages the instrument and poses a safety risk.

2 When the wash is complete, select **Home**.

3 Leave the consumables in place until the next run.
The sippers remain in the SBS and cluster cartridges to prevent air from entering the system. The sippers in the buffer cartridge are raised so that the used reagent bottles can be emptied.

Stagger Runs on Flow Cell A and Flow Cell B

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

1 From the Home screen, select **Sequence**, and then select **A** or **B**.

2 Wait for the run on the adjacent flow cell to pause. To cancel the new run and prevent pausing, select **Cancel**.
If the adjacent run is performing cluster generation, paired-end resynthesis, imaging, or washing, the software completes the current step before pausing.

3 When the adjacent run is paused and the flow cell door opens, set up the new run.
When the new run is started, the paused run is automatically resumed, and then the new run begins.

Appendix A Troubleshooting

Troubleshooting Files

Key File	Folder	Description
Run information file (RunInfo.xml)	Root folder	Contains the run settings: • Number of cycles in the run • Number of reads in the run • Whether the read is indexed • Number of swaths and tiles on the flow cell
Run parameters file (RunParameters.xml)	Root folder	Contains the run name and information about run parameters and run components, including the following RFID information: serial numbers, lot numbers, expiration dates, and catalog numbers.
InterOp files (*.bin)	InterOp	Binary reporting files used for Sequencing Analysis Viewer. InterOp files are updated throughout the run.
Log files	Logs	Log files describe each step performed by the instrument for each cycle, including which reagent is used, and list software and firmware versions used with the run. The file named [InstrumentName]_CurrentHardware.csv lists the serial numbers of instrument components.

Troubleshooting Resources

For technical questions, visit the NovaSeq 6000 Sequencing System support page on the Illumina website. The support page provides access to documentation, downloads, and frequently asked questions. For access to support bulletins, sign in to your MyIllumina account.

For run quality or performance problems, contact Illumina Technical Support. See *Technical Assistance* on page 1. To facilitate troubleshooting, consider sharing a link to the run summary in BaseSpace Sequence Hub with Illumina Technical Support.

Pre-Run Check Errors

If an error occurs during the pre-run checks, use the following actions to resolve it. If you are setting up a dual flow cell run and 1 side fails, you can cancel the failed side and proceed with the side that passed.

When a pre-run check fails, the RFIDs for the flow cell, reagents, and buffers are not locked so the consumables can be used for a subsequent run. When the run is started, the sippers pierce the foil seals on the reagent cartridges and all RFIDs are locked.

System Check	Reason for Failure	Recommended Action
Sensors	A compartment door is open, a consumable is not properly loaded, or at least 1 sensor is not functional.	Select **Retry** and follow the onscreen prompts to resolve the error.
Disk Space	Disk space is insufficient because the specified location of the output folder is full.	Clear disk space from the specified output folder location.

System Check	Reason for Failure	Recommended Action
System Connectivity	The connection to RTA3, the fluidics system, or other connection has been interrupted.	Select **Retry** and follow the onscreen prompts to resolve the error.
Alignment	The position of the flow cell prevents imaging.	Follow the onscreen prompts to reload the flow cell.

Leak Tray

A leak tray is built into the base of the instrument to collect leaked reagents or coolant and collect overflow from the used reagent bottles. Under normal conditions, the leak tray is dry. Leakage indicates a problem with the instrument, and overflow occurs when the used reagent bottles are not regularly emptied.

During the pre-run check, sensors detect whether the leak tray contains any liquids:

- If the leak tray contains liquid but is not full, the run can proceed but you must contact Illumina Technical Support.
- If the leak tray is full, the run cannot proceed and you must contact Illumina Technical Support.

WARNING

Empty the used reagent bottles with ***every run***. Runs are stopped when the small used reagent bottle is full. Overflow from the large used reagent bottle damages the instrument, requires a site visit from an Illumina representative, and poses a safety risk.

Run Failure Before Clustering

If the software fails the run before clustering starts, you can save the reagent cartridges and library tube for a new run. When clustering starts, sippers pierce the foil seals so the reagents and library cannot be used for another run.

You have 2 options for setting up a new run using the reagent cartridges and library tube saved from the failed run:

- **Set up a new run immediately**–Set up the new run within 4 hours of the failed run. The reagent cartridges and library tube remain loaded.
- **Set up a new run later**–Set up the new run within 1 week or 3 weeks of the failed run, depending on storage method. The reagent cartridges and library tube are unloaded from the instrument and stored.

Set Up a New Run Immediately

1. When the run fails, select **Home**.
2. Set up a new run.
3. When prompted, load a new flow cell.
4. Open and close the reagent chiller door and the buffer drawer to prompt the control software to reread the reagent cartridge RFIDs.
   The cartridges and library tube can remain in the instrument for up to 4 hours after the failed run.
5. Empty the used reagent bottles and return them to the instrument.
6. Proceed with run setup.

Set Up a New Run Later

1 When the run fails, select **Home**.

2 Set up a new run or a maintenance wash to release the consumables from the instrument.

3 When prompted, remove and store the following consumables:
- Cap the library tube and store at 2°C to 8°C for up to 1 week ***or*** at -25°C to -15°C for up to 3 weeks. Do not mix storage methods.
- Return the SBS and cluster cartridges to -25°C to -15°C storage.
- Return the buffer cartridge to room temperature storage protected from light.

4 Select **End** to cancel the run or maintenance wash, and then select **Yes** to confirm the command.

End a Run

Ending a run on the NovaSeq 6000 system is ***final***. The software cannot resume the run or save sequencing data, and consumables cannot be reused.

If you end the run between clustering completion and Read 1 completion, the software displays wash options. Otherwise, the software initiates the automatic post-run wash.

1 Select **End**, and then select **Yes** to confirm the command.
If the run was ended after Read 1, the software initiates the automatic post-run wash.

2 If prompted, select from the following wash options:
- **End Run Without Wash**–End the run and initiate a maintenance wash.
- **End Run and Wash**–End the run and perform an automatic post-run wash.
- **Cancel**–Continue with the current run.

3 If you selected End Run Without Wash, follow the software prompts to set up a maintenance wash.

Shut Down the Instrument

Shutting down the instrument safely shuts down all software and systems, and turns off instrument power. The status bar fades from green to white, indicating that the shutdown is in progress.

Under normal circumstances, shutting down the instrument is unnecessary.

1 From the Main Menu, select **Shutdown Instrument**.
Wait at least 60 seconds before turning on the instrument again.

CAUTION

Do not relocate the instrument. Improper moving can affect optical alignment and compromise data integrity. For relocation assistance, contact your Illumina representative.

Appendix B Real-Time Analysis

Real-Time Analysis Overview

The NovaSeq 6000 Sequencing System runs RTA3, an implementation of Real-Time Analysis software, on the instrument Compute Engine (CE). RTA3 extracts intensities from images received from the camera, performs base calling, assigns a quality score to base calls, aligns to PhiX, and reports data in InterOp files for viewing in Sequencing Analysis Viewer.

To optimize processing time, RTA3 stores information in memory. If RTA3 is terminated, processing does not resume and run data are not saved.

RTA3 Inputs

RTA3 requires tile images contained in local system memory for processing. RTA3 receives run information and commands from the control software.

RTA3 Outputs

Images for each color channel are passed in memory to RTA3 as tiles. From these images, RTA3 outputs a set of quality-scored base call files and filter files. All other outputs are supporting output files.

File Type	Description
Base call files	Each tile that is analyzed is included in a concatenated base call (*.cbcl) file. Tiles from the same lane and surface are aggregated into 1 *.cbcl file for each lane and surface.
Filter files	Each tile produces a filter file (*.filter) that specifies whether a cluster passes filters.
Cluster location files	Cluster location (*.locs) files contain the X,Y coordinates for every cluster in a tile. A cluster location file is generated for each run.

Output files are used for downstream analysis in BaseSpace Sequence Hub. Alternatively, use bcl2fastq conversion software for FASTQ conversion and third-party analysis solutions. NovaSeq files require bcl2fastq Conversion Software v2.19, or later. For the latest version of bcl2fastq, visit the NovaSeq 6000 Sequencing System downloads page on the Illumina website.

RTA3 provides real-time metrics of run quality stored as InterOp files, which are a binary output containing tile, cycle, and read-level metrics. Viewing real-time metrics using Sequencing Analysis Viewer requires InterOp files. For the latest version of Sequencing Analysis Viewer, visit the Sequencing Analysis Viewer downloads page on the Illumina website.

Error Handling

RTA3 creates log files and writes them to the Logs folder. Errors are recorded in a text file in *.log file format.

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

- info_00000.log summarizes important run events.
- error_00000.log lists errors that occurred during a run.
- warning_00000.log lists warnings that occurred during a run.

Flow Cell Tiles

Tiles are small imaging areas on the flow cell. The camera takes a single image of each swath, which the software divides into tiles for RTA3 processing. The total number of tiles depends on how many lanes, swaths, and surfaces are imaged on the flow cell.

The S2 flow cell has a total of 1408 tiles. Each of the 2 lanes has 4 swaths per surface with 88 tiles per swath. Both surfaces of the flow cell are imaged for a total of 2 surfaces.

Table 2 Flow Cell Tiles

Flow Cell Component	S2	Description
Lanes	2	A lane is a physical channel with input and output ports.
Surfaces	2	The flow cell is imaged on 2 surfaces: the top and bottom. The top surface of a tile is imaged first.
Swaths per lane	4	A swath is a column in a flow cell lane that the camera captures as 1 image.
Tiles per swath	88	A tile is a portion of a swath and depicts an imaged area on the flow cell.
Total tiles generated	1408	Lanes × surfaces × swaths × tiles per swath equals the total number of tiles.

Tile Naming

The tile name is a 5-digit number that represents the position on the flow cell.

- The first digit is the lane number: 1 or 2.
- The second digit represents the surface: 1 for top or 2 for bottom.
- The third digit represents the swath number: 1, 2, 3, or 4.
- The last 2 digits represent the tile number, 01 through 88. Tile numbering starts with 01 at the outlet end of the flow cell through 88 at the inlet end.

For example, tile name 1_1205 indicates lane 1, top surface, swath 2, tile 5.

Real-Time Analysis Workflow

Registration — Records the location of each cluster on the patterned flow cell.

↓

Intensity extraction — Determines an intensity value for each cluster.

↓

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.

Registration

Registration aligns an image to the hexagonal array of nanowells on the patterned flow cell. Because of the ordered arrangement of nanowells, the X and Y coordinates for each cluster in a tile are predetermined. Cluster positions are written to a cluster location (s.locs) file for each run.

If registration fails for any images in a cycle, no base calls are generated for that tile in that cycle. Use Sequencing Analysis Viewer to identify which images failed registration.

Intensity Extraction

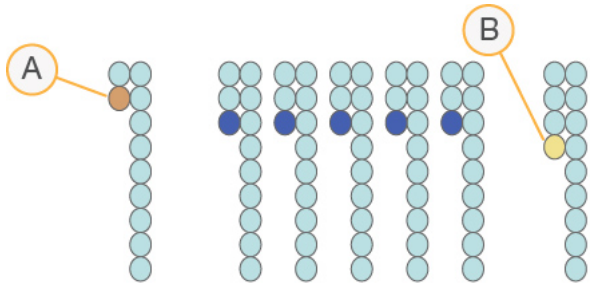
After registration, intensity extraction calculates an intensity value for each nanowell in a given image. If registration failed, the intensity for that tile cannot be extracted.

Phasing Correction

During the sequencing reaction, each DNA strand in a cluster extends by 1 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 23 Phasing and Prephasing



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

RTA3 corrects the effects of phasing and prephasing, which maximizes the data quality at every cycle throughout the run.

Base Calling

Base calling determines a base (A, C, G, or T) for every cluster of a given tile at a specific cycle. The NovaSeq 6000 Sequencing System uses 2-channel sequencing, which requires only 2 images to encode the data for 4 DNA bases, 1 from the red channel and 1 from the green channel.

A no call is identified as N. No calls occur when a cluster does not pass filter, registration fails, or a cluster is shifted off the image.

Intensities extracted from the images in 2 channels are compared to another image result in 4 distinct populations, each corresponding to a nucleotide. The base calling process determines which population each cluster belongs to.

Figure 24 Visualization of Cluster Intensities

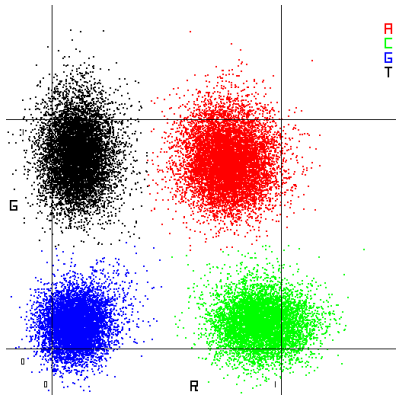


Table 3 Base Calls in 2-Channel Sequencing

Base	Red Channel	Green Channel	Result
A	1 (on)	1 (on)	Clusters that show intensity in both the red and green channels.
C	1 (on)	0 (off)	Clusters that show intensity in the red channel only.
G	0 (off)	0 (off)	Clusters that show no intensity at a known cluster location.
T	0 (off)	1 (on)	Clusters that show intensity in the green channel only.

Clusters Passing Filter

During the run, RTA3 filters raw data to remove reads that do not meet the data quality threshold. Overlapping and low-quality clusters are removed.

For 2-channel analysis, RTA3 uses a population-based system to determine the chastity (intensity purity measurement) of a base call. Clusters pass filter (PF) when no more than 1 base call in the first 25 cycles has a chastity below a fixed threshold. PhiX alignment is performed at cycle 26 on a subset of tiles for clusters that passed filter. Clusters that do not pass filter are not base called and not aligned.

Quality Scores

A quality score (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. After the Q-score is determined, results are recorded in base call (*.cbcl) files.

The Q-score succinctly communicates small error probabilities. Quality scores are represented as Q(X), where X is the score. The following table shows the relationship between a quality score and error probability.

Q-Score Q(X)	Error Probability
Q40	0.0001 (1 in 10,000)
Q30	0.001 (1 in 1000)
Q20	0.01 (1 in 100)
Q10	0.1 (1 in 10)

Quality Scoring

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.

NOTE

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

Quality Score Binning

RTA3 assigns each base call 1 of 3 quality scores based on the confidence of the base call. This Q-score binning reduces storage space and bandwidth requirements without affecting accuracy or performance.

For more information on Q-score resolution, see *Reducing Whole-Genome Data Storage Footprint (Pub. No. 970-2012-013)*.

Appendix C Output Folders and Files

Sequencing Output Folder Structure

The control software generates the output folder name automatically.

- **Config**–Configuration settings for the run.
- **Logs**–Log files describing operational steps, instrument analytics, and RTA3 events.
- **Data**
  - **Intensities**
    - **BaseCalls**
      - **L00[X]**–Base call files (*.cbcl) aggregated in 1 file per lane, surface, and cycle.
    - s.locs–The cluster locations file for the run.
- **InterOp**–Binary files used by Sequencing Analysis Viewer.
- **Recipe**–Run-specific recipe file.
- **Thumbnail Images**–Thumbnail images for every 10th tile.
- **LIMS**–The run setup file (*.json), if applicable.
- RTA3.cfg
- RunInfo.xml
- RunParameters.xml
- RTAComplete.txt
- CopyComplete.txt
- Samplesheet.csv–Sample sheet or other attached file, if applicable.
- SequenceComplete.txt

Sequencing Output Files

File Type	File Description, Location, and Name
Base call files	Each cluster analyzed is included in a base call file, aggregated in 1 file per cycle, lane, and surface. The aggregated file contains the base call and encoded quality score for every cluster. The base call files are used by BaseSpace Sequence Hub or bcl2fastq2. Data\Intensities\BaseCalls\L001\C1.1 L[lane]_[surface].cbcl, for example L001_1.cbcl
Cluster location files	For each flow cell, a binary cluster location file contains the XY coordinates for clusters in a tile. A hexagonal layout that matches the nanowell layout of the flow cell predefines the coordinates. Data\Intensities s_[lane].locs
Filter files	The filter file specifies whether a cluster passed filters. Filter files are generated at cycle 26 using 25 cycles of data. For each tile, 1 filter file is generated. Data\Intensities\BaseCalls\L001 s_[lane]_[tile].filter

File Type	File Description, Location, and Name
InterOp files	Binary reporting files used for Sequencing Analysis Viewer. InterOp files are updated throughout the run. InterOp folder
Run information file	Lists the run name, number of cycles in each read, whether the read is an Index Read, and the number of swaths and tiles on the flow cell. The run info file is created at the beginning of the run. [Root folder], RunInfo.xml
Thumbnail files	When enabled, a thumbnail image for every 10th tile in each color channel (red and green). Thumbnail_Images\L001\C[X.1]–Files are stored in a subfolder for each cycle. s_[lane]_[tile]_[channel].jpg–The thumbnail image includes the tile number.

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