Genome Analysis
User Guide

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ILLUMINA PROPRIETARY
Part # 11251649

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Notice

Revision History

Revision Letter	Date
A	July 2007

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Introduction

The Illumina Genome Analysis System is a groundbreaking new platform for genetic analysis and functional genomics. Dramatically improving speed and reducing costs, it is suitable for a range of applications including whole genome and candidate region sequencing, expression profiling, and small RNA identification and quantitation. Leveraging proprietary reversible terminators and Clonal Single Molecule Array technology, the Illumina Genome Analysis System has the potential to generate upwards of one billion bases of data per run, and in the process transform the way many experiments are devised and carried out.

The Illumina Genome Analysis System is ideal for genome-scale as well as targeted sequencing projects. This platform has the potential to allow researchers to sequence a human genome for under $100,000 and in a matter of weeks, a feat that marks a dramatic improvement over the capabilities offered by existing technologies.

Sequencing-By-Synthesis (SBS), using proprietary reversible terminators, lets the Illumina Genome Analysis System provide a high degree of sequencing accuracy even through homopolymeric regions. This allows researchers to sequence complex genomes rapidly and economically. The versatile format of the flow cell also enables researchers to tailor the system to meet the specific needs of their application.

Audience and Purpose

This guide is for laboratory personnel and other individuals responsible for:

- Operating the Illumina Cluster Station and Genome Analyzer
- Maintaining instrument components and consumables
- Assessing data quality with Run Browser

This guide also provides background information about core concepts such as recipes, sample sheets, and run folders. The chapter on frequently asked questions provides additional support.

Other SBS Documentation

The following is a list of available documentation. Please consult with Illumina Technical Support to find out about recent updates and releases of new documents such as additional sample prep protocols.

Table 1 *Documentation*

Guide	Illumina Part #	Description
Cluster Station Reagent Prep Guide	11251606	Information about how to prepare reagents for cluster generation, including a shipment checklist, guidance on how to load the reagents, and instructions on how to prepare certain reagents for use. This information does not apply to Paired End sample prep.
Cluster Station Site Preparation Guide	11251665	Information about how to prepare your lab for the Cluster Station, including environmental requirements, lists of user-supplied consumables, and safety hazards.
*Dpn*II *GEX Sample Prep Guide*	11251729	Information about how to prepare gene expression tag samples for sequencing using *Dpn*II digestion.
gDNA Sample Prep Guide	11251892	Information about how to prepare genomic DNA samples for sequencing.
Genome Analysis Lab Tracking Worksheet	11251817	Printable forms where lab technicians can record lot numbers, operator names, and other information for each run.
Genome Analyzer Reagent Prep Guide	11251622	Information about how to prepare reagents for sequencing, including a shipment checklist, guidance on how to load the reagents, and instructions on how to prepare certain reagents for use. This information does not apply to Paired End sample prep.
Genome Analyzer Site Preparation Guide	11251681	Information about how to prepare your lab for the Genome Analyzer, including environmental requirements, lists of user-supplied consumables, and safety hazards.
*Nla*III *GEX Sample Prep Guide*	11251702	Information about how to prepare gene expression tag samples for sequencing using *Nla*III digestion.
Paired End Sequencing User Guide	11251753	Information about paired-end reagent prep, cluster generation, and sequencing. This guide assumes that you are familiar with the contents of the *Genome Analysis User Guide*.

Illumina Genome Analysis

The Genome Analysis process is straightforward yet flexible, consisting of four steps:

1. Sample preparation. This is the only step that involves significantly different procedures for different types of samples.
2. Cluster generation on the Cluster Station.
3. Sequencing by Synthesis on the Genome Analyzer.
4. Data analysis using the Analysis Pipeline software.

Sample Prep

Sequencing by Synthesis can be used for multiple applications, including DNA sequencing, small RNA studies, and gene expression. While the process of generating clusters and analyzing them is standardized across all applications, the process of preparing samples is unique to each application. For instructions on preparing samples for your current application, see the appropriate sample prep booklet.

Cluster Station

The Cluster Station is a hardware device that hybridizes samples onto a flow cell and amplifies them for later sequencing on the Genome Analyzer. During cluster creation, a single DNA fragment (the template) is attached to the surface of a specially coated flow cell and amplified to form a surface-bound colony (the cluster). You can seed the surface of a single flow cell with millions of different DNA fragments. The result is a heterogeneous population of clusters, with each cluster consisting of many identical copies of the original template molecule.

Genome Analyzer

Using a massively parallel sequencing approach, the Illumina Genome Analyzer can generate more than one billion bases of data in a single run. The system leverages Solexa sequencing technology and novel reversible terminator chemistry, optimized to achieve unprecedented levels of cost effectiveness and throughput.

Flow Cell

The flow cell is a multi-channel glass and silicon-based substrate in which clusters are generated and the sequencing reaction is performed. Each of the channels are individually addressable, so researchers can interrogate multiple distinct samples per flow cell. Within each channel of the flow cell, millions of primers act as capture mechanisms for the fragmented DNA or cDNA. Each channel of the flow cell is capable of yielding up to 5 million distinct clusters and generating over 125Mb of sequence data. The versatile format of the flow cell allows researchers to tailor the use of the device to the specific needs of their applications and use the platform for a variety of interrogations.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 2 *Illumina Technical Support Contacts*

Contact	Number
Toll-free Customer Hotline	1-800-809-ILMN (1-800-809-4566)
International Customer Hotline	1-858-202-ILMN (1-858-202-4566)
Illumina Website	www.illumina.com

Chapter 2

Using the Cluster Station

Topics

Introduction

The Cluster Station is a hardware device that hybridizes samples onto a flow cell and amplifies them for later sequencing on the Genome Analyzer. It uses solid support amplification to create an ultra-high density sequencing flow cell with > 10 million clusters, each containing ~1,000 copies of template per square centimeter. The Cluster Station works in conjunction with a dedicated workstation that runs the Illumina Cluster Station software.

The Cluster Station is fully automated. After loading the reagents and flow cell, you can walk away from the system. Alternatively, our open-source software allows you to run individual subroutines or modify protocols to meet your research needs.

The Cluster Station dispenses reagents and controls reaction times, flow rates, and temperatures. The flow rate and the temperature of the flow cell are computer-controlled, using syringe pumps and a Peltier block.

Figure 1 Cluster Station

Cluster Generation Steps

Cluster generation consists of the following steps:

1. **Hybridize template DNA**—Hybridize template molecules onto the surface of the flow cell.
2. **Amplify template DNA**—Isothermally amplify the molecules to generate dsDNA clusters.
3. **Linearize**—Chemically linearize the dsDNA clusters. This is the first step of converting dsDNA to ssDNA that is suitable for sequencing.

4. **Block**—Block the free 3' OH ends of the linearized dsDNA clusters. This prevents nonspecific sites from being sequenced. After this recipe, the flow cell is stable and can be stored.
5. **Denature**—This finishes converting the dsDNA to ssDNA.
6. **Hybridize sequencing primers**—Hybridize a sequencing primer, or multiple sequencing primers, onto the linearized and blocked clusters. After this step, the flow cell is ready for sequencing.

Cluster Station Recipes

The Cluster Station workstation is preloaded with several protocol recipes. Most of them perform different subsets of the same protocol, so that you have flexibility in scheduling your work (see *Protocol Times* on page 10). The default location is <install directory>\DataCollection_v<#>\bin\Recipes. The <#> in the filename refers to the current version of the recipe. To learn more about recipes, see Appendix C, *Recipes*.

A typical workflow uses the following two recipes:

1. Amplification_Linearization_Blocking_v<#>
2. Primerhyb_only_v<#>

At the beginning of each recipe, you must load the instrument with fresh reagents and set up the automatic run. At the end of each run, the Cluster Station performs a washing step.

The following table lists the tasks associated with each Cluster Station recipe.

Table 3 Tasks in Each Cluster Station Recipe

Recipe	Hybridize and Amplify Template DNA	Linearize	Block	Denature DNA and Hybridize Sequencing Primer(s)
Amplification_only_v<#>	X			
Linearization_Blocking_Primerhyb_v<#>		X	X	X
Amplification_Linearization_Blocking_v<#>	X	X	X	
Linearization_Blocking_only_v<#>		X	X	
Primerhyb_only_v<#>				X
Multi_Primerhyb_v<#>				X
One_step_full_protocol_v<#>	X	X	X	X

Protocol Times

This table shows approximately how long the Cluster Station takes to perform each step of the clustering protocol. You must wash the amplification lines before running this protocol. For more instructions, see *WashAmponlyLines* on page 28.

Aside from the One Step recipe, all Cluster Station recipes perform a subset of the overall procedure.

Step	Duration	Solution Change	Reagent (Position)	Time for Reagent
Template Hybridization and Initial Extension	38 min	1	Hybridization Buffer (A)	2 min
		2	Template Mix (B)	25 min
		3	Wash Buffer (C)	5 min
		4	Amplification Pre-Mix* (D)	3 min 20 s
		5	Initial Extension Mix with *Taq* Polymerase* (E)	3 min
Isothermal Amplification	1 hr 47 min (35 cycles)	6	Formamide (9)	56 s
		7	Amplification Pre-Mix* (11)	56 s
		8	Amplification Mix with *Bst* Polymerase* (1)	72 s
			Storage Buffer (12)	
Safe Stopping Point				
Linearization	25 min	9	Linearization Solution* (3)	20 min
		10	Water (14)	5 min
Blocking	43 min	11	1X Blocking Buffer * (15)	5 min
		12	Blocking Mix * (5)	33 min
		13	Wash Buffer (10)	5 min
			Storage Buffer (12) *Only occurs in recipes that end with the blocking step*	
Safe Stopping Point				
Denaturation and Hybridization of Sequencing Primer(s)	35 min	14	NaOH (17)	5 min
		15	TE (18)	5 min
		16	Sequencing Primer Mix* (7)	20 min
		17	Wash Buffer (10)	5 min
			Storage Buffer (12)	

Step	Duration	Solution Change	Reagent (Position)	Time for Reagent
Ready for sequencing. Do not store flow cell.				
Total Time	≈ 4 h 8 min	* These solutions are made fresh using kit reagents		

Scheduling

This flow chart shows some of the ways you can use recipes to adapt the assay schedule to your needs.

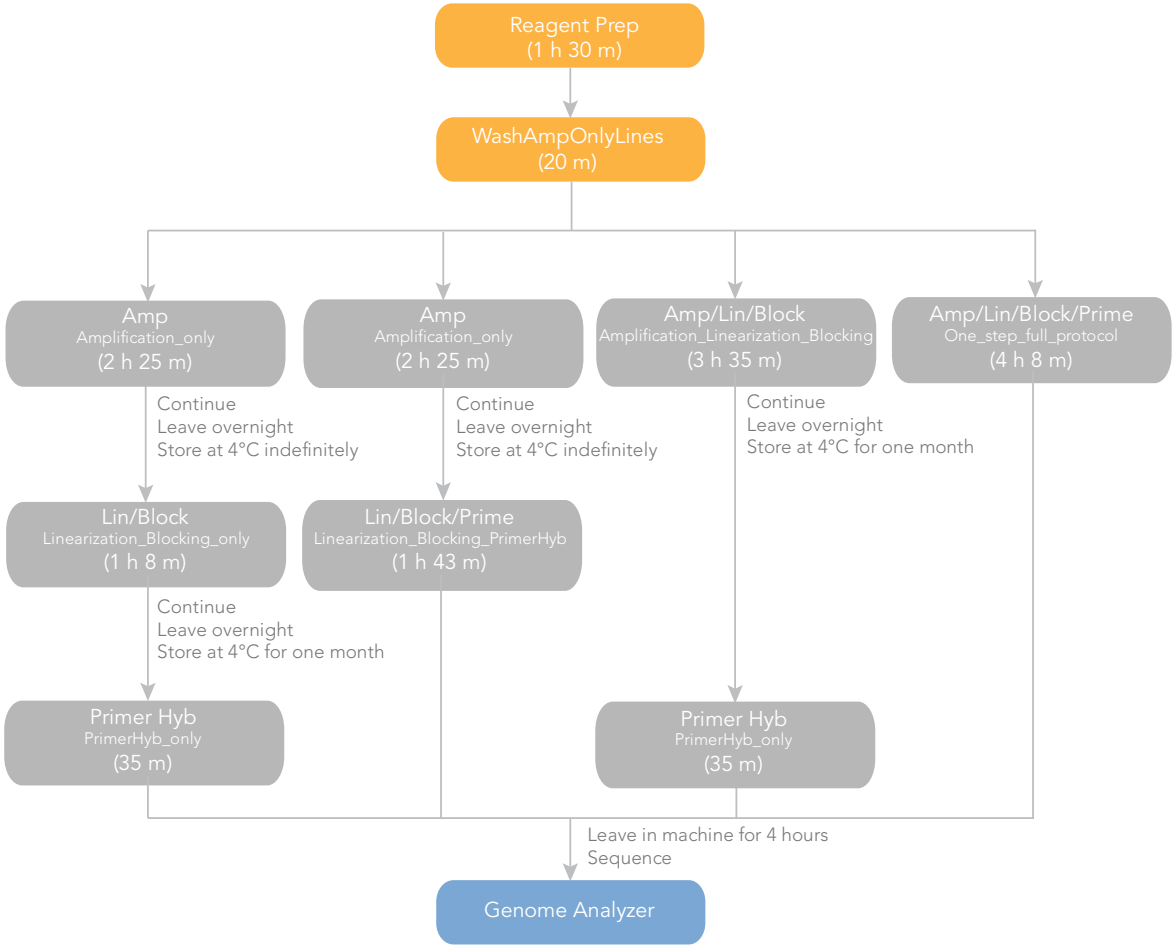

Figure 2 Scheduling the Assay

Whenever you stop for the day, you must run the WashFullProtocolLines recipe. For instructions, see *WashFullProtocolLines* on page 28. Normally, the recipe prompts you to perform this wash. If you are continuing immediately to the next recipe (for example, from Linearization_Blocking_only to PrimerHyb_only), you do not need to perform the wash.

As a best practice, run amplification and/or linearization and blocking on a number of flow cells and store them at 4°C as instructed in *Safe Stopping Points During Cluster Generation* on page 26. Then, if a flow cell does not pass first-base incorporation in the Genome Analyzer, you can sequence one of the backup flow cells and not waste either the machine time or the reagents.

Components

Power Connections

Place the instrument at least six inches away from the wall so that you can easily reach the power switch, universal power input, and USB connection on the back of the Cluster Station.

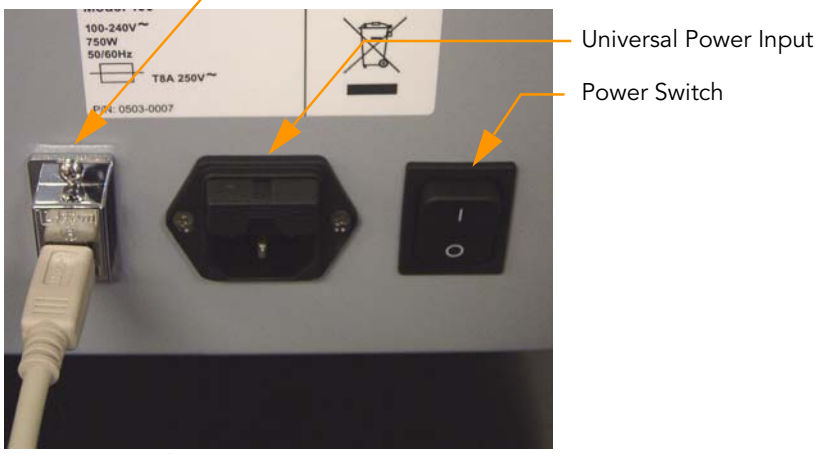

Figure 3 Cluster Station Power Connections

Instrument Areas

All operator activity on the instrument occurs in two main compartments:

- Reagent Area
- Flow Cell Area

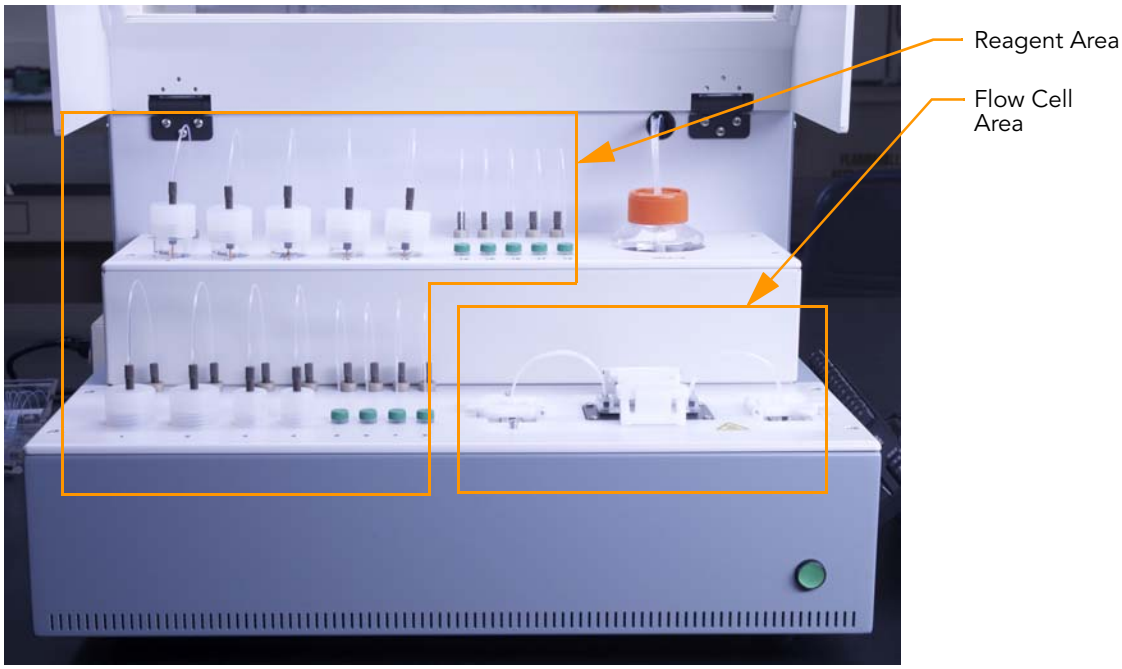

Figure 4 Cluster Station Areas

Reagent Area

The reagent area holds reagent tubes in various sizes, a removable strip tube holder, and a waste container. Each reagent position has a unique number associated with it, and each strip tube has a unique letter. When you prepare reagents, you will be asked to place the containers in the appropriate numbered or lettered location.

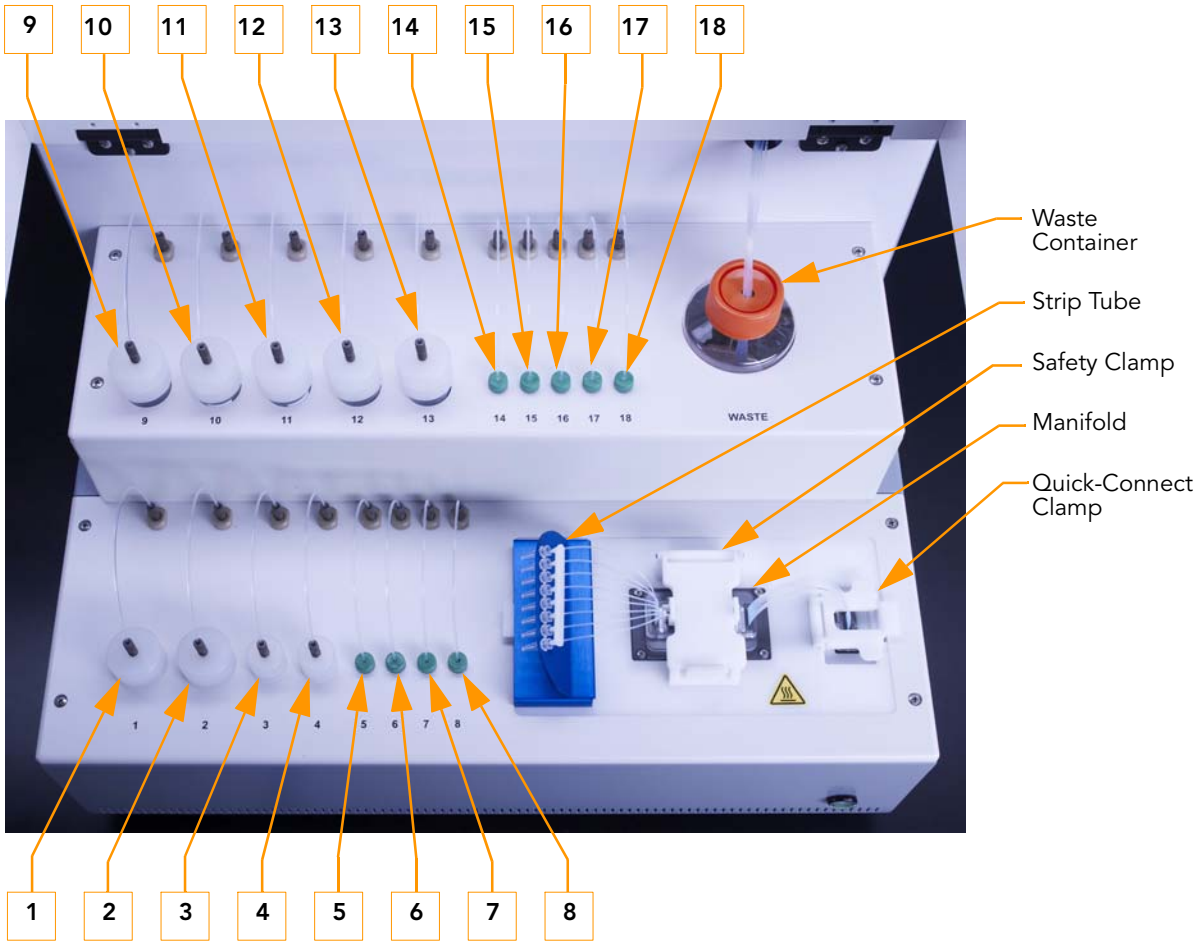

Figure 5 Cluster Station with Numbered Reagent Bottles

Waste Container

The 250 ml waste container catches the reagents after they have gone through the flow cell and/or manifolds. It is located in the upper right corner of the Cluster Station. Empty the waste container after every run. Always check the waste level in the waste container before starting a run to ensure that it is empty.

Figure 6 Liquid Waste Container on Cluster Station

Fluid Handling Lines

The inside diameter of all lines on the Cluster Station is 0.5 mm. There is about 400 µl of space in the lines between the reagent bottles and the waste container.

Syringes and pumps pull fluid through the system. The suction is not strong enough to dissipate all of the air bubbles, so it is important to keep water in the lines at all times.

Disposable hybridization or amplification manifolds are connected to either side of the flow cell, so that they can conduct reagents from the strip tubes or reagent bottles, respectively, into the flow cell. The removable strip tube holder holds eight 0.2 ml strip tubes for samples and reagents.

Flow Cell Area Components

The flow cell is placed on the flow cell stage at the front right corner of the Cluster Station. A white safety clamp holds the flow cell and manifold in place.

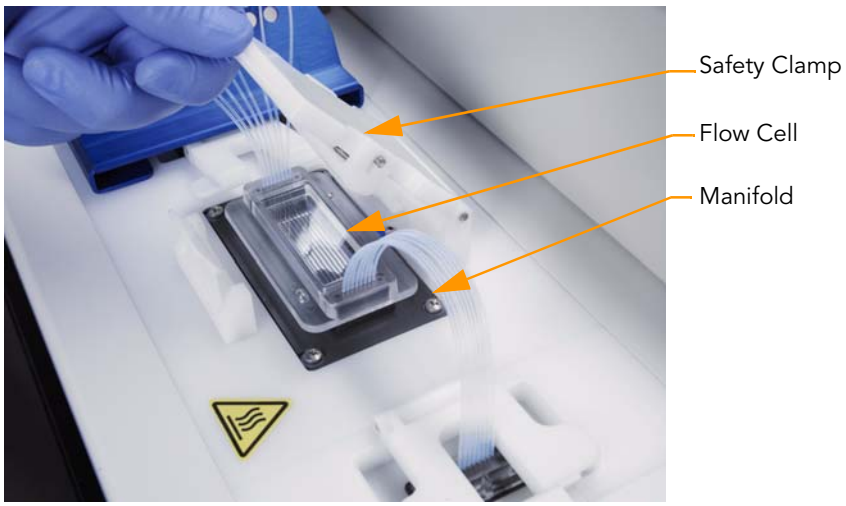

Figure 7 Flow Cell Area Components

Manifolds

Hybridization Manifold

The Hybridization Manifold is a disposable item used for sample loading and hybridization. It enables the Cluster Station to transfer reagents from the individual wells of the eight 0.2 ml strip tubes into the flow cell. Up to eight different samples can be loaded and hybridized in parallel. To prevent cross-contamination, use each manifold only once.

Insert the Removable Strip Tube Holder whenever you use the Hybridization Manifold. Insert the fanned-out tubes on one end of the hybridization manifold into the strip tubes, and connect the grouped tubes on the other side to the output port, which flows into the waste container.

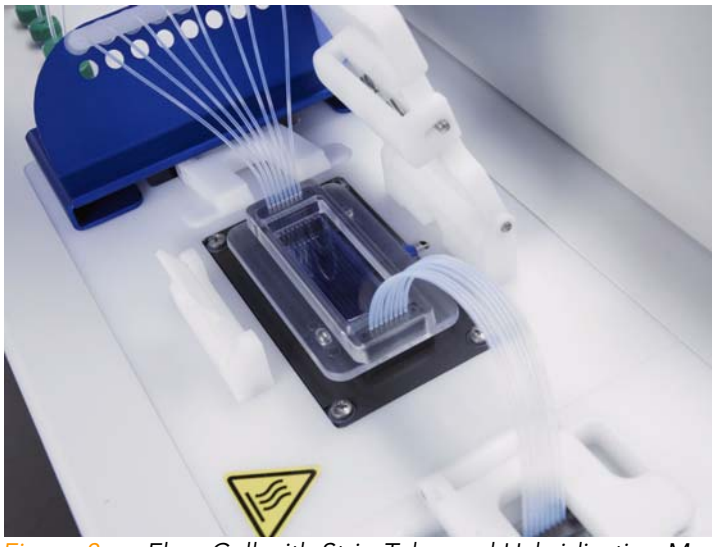

Figure 8 Flow Cell with Strip Tube and Hybridization Manifold

Amplification Manifold

The Amplification Manifold is a disposable item used for all steps after template hybridization: Amplification, Linearization, Blocking, Denaturation, and Hybridization of Sequencing Primer. It enables the Cluster Station to transfer reagents from the Cluster Station into the flow cell in the proper order.

To prevent cross contamination, use each manifold only once.

Attach the Amplification Manifold whenever prompted by the Cluster Station software. Connect the tubes on one end to the input port, which draws reagents from the reagent positions on the Cluster Station. Connect the tubes on the other side to the output port, which flows into the waste container. Make sure the tubes are securely connected.

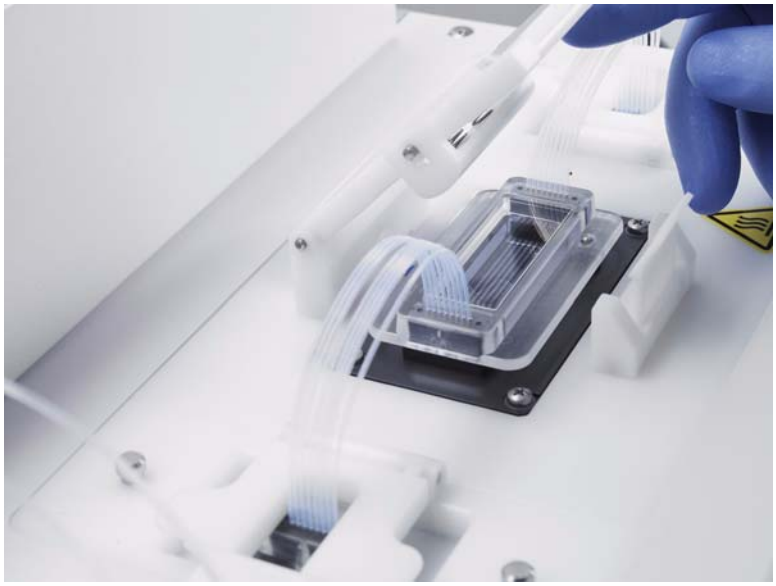

Figure 9 Flow Cell with Amplification Manifold

Quick-Connect Clamps

The quick-connect clamps by the input and output ports enable you to snap the tubes on the amplification and hybridization manifolds into place.

The bottom of the quick-connect clamp is fitted with a rubber gasket. The gasket creates a constant tight seal to prevent leakage. If you notice a loose gasket, tighten the seal by pushing the gasket back into the quick connect.

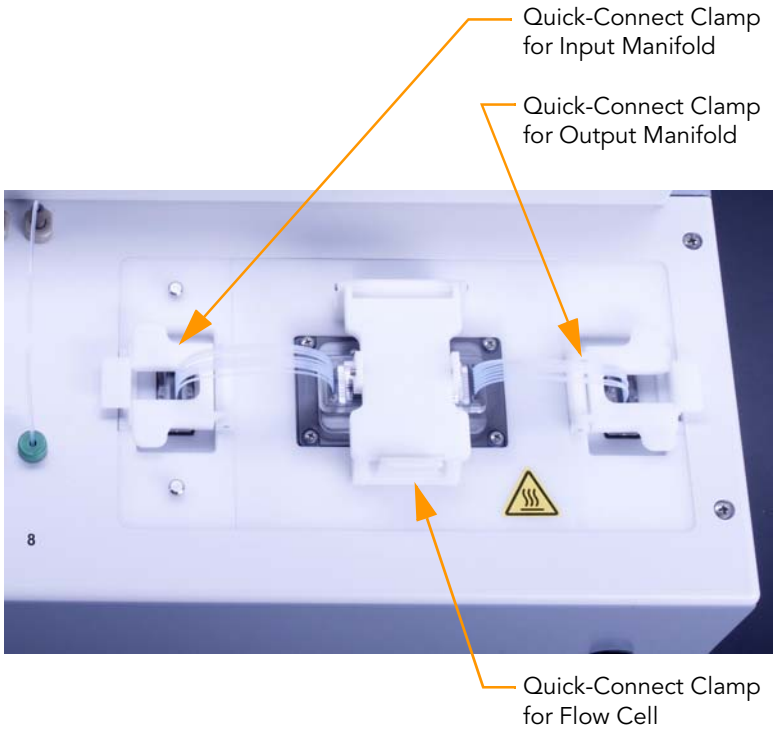

Figure 10 Quick-Connect Clamps

Washing Bridge

The washing bridge is a reusable manifold used during instrument washes. It connects the input manifold directly to the output manifold, skipping over the flow cell. This allows you to flush all the reagent lines with water at the conclusion of a cluster generation protocol.

Figure 11 Washing Bridge

Input Manifold

The input manifold is the port that reagents pass through to get to the flow cell. It is located to the left of the flow cell stage as you face the Cluster Station. One set of tubes from the amplification manifold plugs into this port and is held in place by the quick connect clamp.

Output Manifold

The output manifold is the port that receives the liquid flowing out of the flow cell and transfers it to the waste container. The amplification manifold, hybridization manifold, and washing bridge all connect to the output manifold.

Cluster Station Workflow

Table 4 Cluster Generation Process

Step	Instructions
1. Ensure that you have all of the required user-supplied equipment and consumables.	*Cluster Station Site Preparation Guide* (Illumina part # 11251665)
2. Restart the Cluster Station and attached workstation.	*Starting the Cluster Station* on page 19
3. Prepare fresh reagents.	*Preparing Reagents for the Cluster Station* (Illumina part # 11251606)
4. Load the reagents in their appropriate positions on the Cluster Station.	*Loading Reagents* on page 20 for best practices *Preparing Reagents for the Cluster Station* (Illumina part # 11251606) for specific amounts and positions
5. Load the flow cell into the Cluster Station.	*Loading the Flow Cell* on page 23
6. Open and run a recipe.	*Running a Recipe* on page 24
7. After hybridizing the sequencing primer(s), sequence the flow cell within 4 hours.	Chapter 3, *Using the Genome Analyzer*

Starting the Cluster Station

Illumina recommends that you reboot the Cluster Station computer once or twice a week.

It is important to turn on the Cluster Station *before* starting the software. Otherwise, the software will not control the Cluster Station.

1. Turn the main power switch on the Cluster Station to the ON position.
2. Wait for 20 seconds.
3. Start the computer and log on. The default logon is:

   Username: sbsuser

   Password: sbs123

   If the default logon does not work, check with your IT personnel to find out the correct user name and password for your site.
4. Double-click the Illumina Cluster Station software icon on the computer desktop to launch the software.

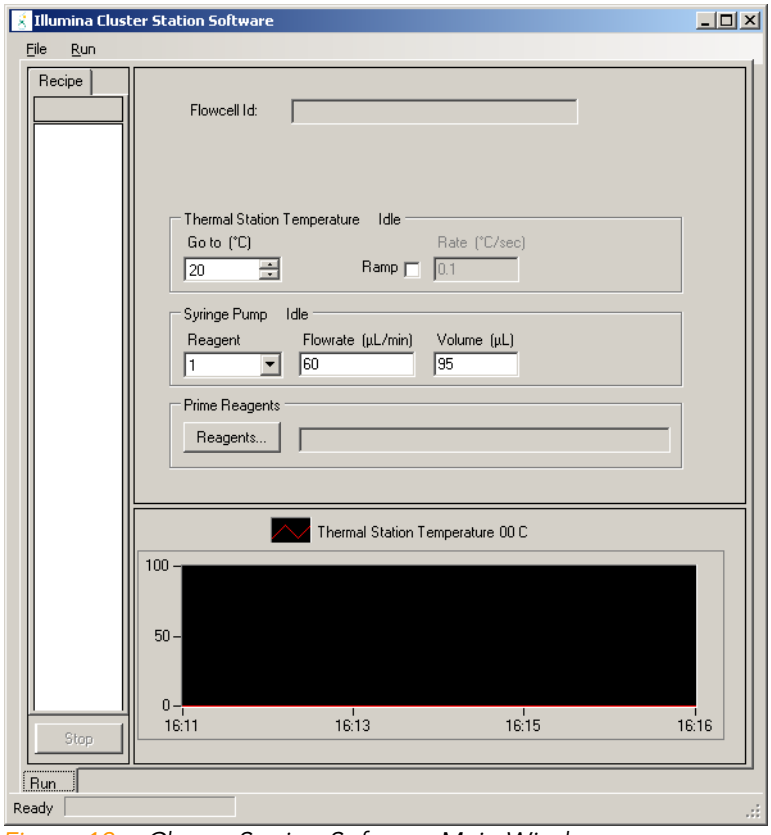

Figure 12 Cluster Station Software Main Window

Preparing Reagents

For instructions on preparing reagents, refer to *Preparing Reagents for the Cluster Station* (Illumina part # 11251606). Prepare all the reagents as described before proceeding.

Loading Reagents

To prevent cross-contamination, follow these best practices:

- Always remove and replace instrument reagents one tube (or bottle) at a time.
- Wear gloves at all times. Do not touch reagents with bare hands.
- Connect the 50 ml, 15 ml, and 1.5 ml tubes by holding the caps stationary while you twist the tubes into place. This prevents crimping and twisting of the lines. Then, place the capped tubes into the instrument.

The reagent compartment holds three sizes of tubes:

- 50 ml conical bottom tubes
- 15 ml conical bottom tubes
- 1.5 ml tubes

Not all 15 ml tubes fit the Cluster Station. Illumina recommends BD Falcon, Catalog # 352096 or 352097.

The following figure illustrates accurate reagent tube and bottle placement along with the number associated with each position. The strip tubes that fit in the removable strip tube holder are lettered from A to J.

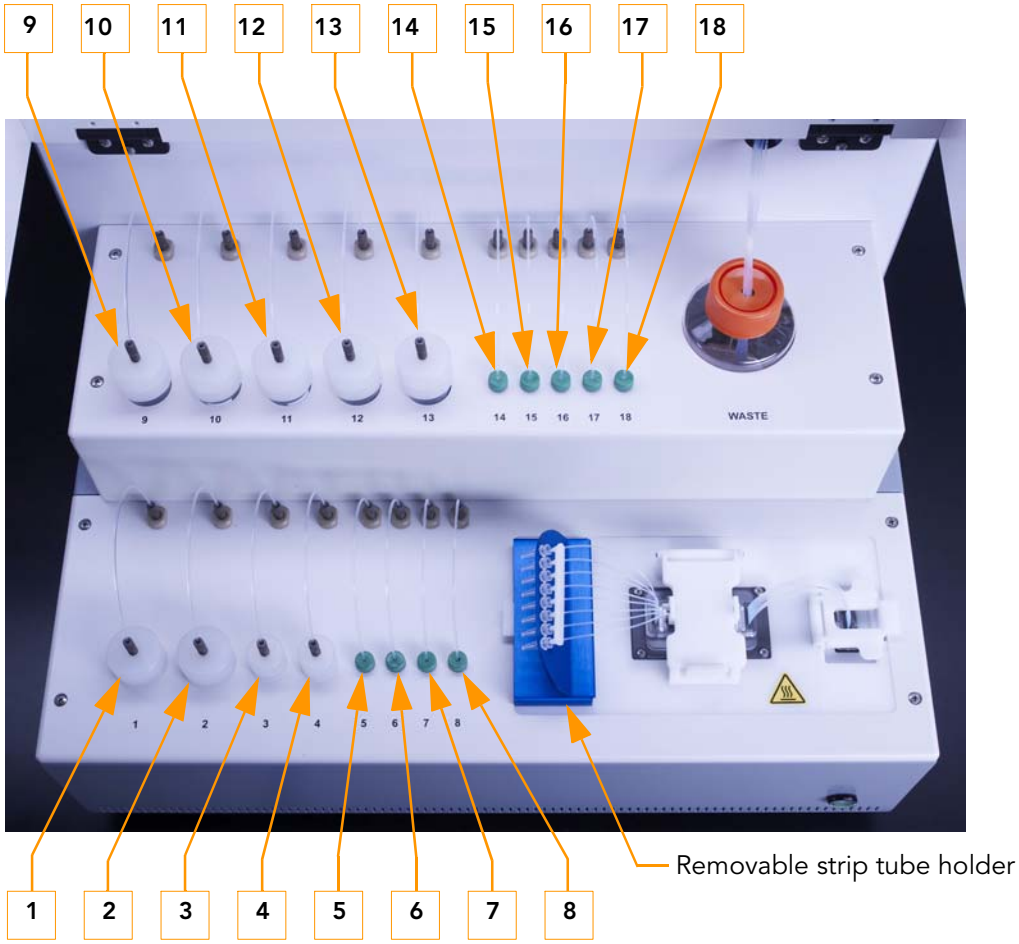

Figure 13 Reagent Positions

Table 5 *Reagent Positions*

Position	Reagent	Tube Size
1	Amplification Mix with *Bst* DNA Polymerase	50 ml
2	*Spare*	50 ml
3	Linearization Mix	15 ml
4	*Spare*	15 ml
5	Blocking Mix	1.5 ml
6	*Spare*	1.5 ml
7	Primer Mix	1.5 ml
8	*Spare*	1.5 ml
9	Formamide	50 ml
10	Wash Buffer	50 ml
11	Amplification Pre-Mix	50 ml
12	Storage Buffer	50 ml
13	*Spare*	50 ml
14	Deionized Water	1.5 ml
15	1x Blocking Buffer	1.5 ml
16	*Spare*	1.5 ml
17	0.1N NaOH	1.5 ml
18	TE	1.5 ml
A	Hybridization Buffer	0.2 ml eight-strip tube
B	Template Mix	0.2 ml eight-strip tube
C	Wash Buffer	0.2 ml eight-strip tube
D	Amplification Pre-Mix	0.2 ml eight-strip tube
E	Initial Extension Mix with *Taq* Polymerase	0.2 ml eight-strip tube
F	0.1N NaOH	0.2 ml eight-strip tube
G	TE	0.2 ml eight-strip tube
H	Primer Mixture	0.2 ml eight-strip tube
I	Wash Buffer	0.2 ml eight-strip tube
J	Storage Buffer	0.2 ml eight-strip tube

Loading the Flow Cell

Always use clean gloves or plastic forceps when handling the flow cell. Do not touch the flow cell with bare hands or marker pens. Doing so can leave marks that could interfere with the detection of clusters.

1. Remove a flow cell from its case.
2. Wipe and dry the flow cell using lens cleaning tissue.

   This step ensures that the flow cell does not stick to the platform. Be careful not to drain the channels when wiping the ports (holes).
3. Make sure that the stage is clean and free from dust and salt.
4. Place the flow cell on the thermal block of the Cluster Station with the ports facing up. The arrow etched on the flow cell should face up at the top left corner.
5. Take a new hybridization manifold from a sealed bag.

CAUTION Always shield the manifold gaskets from contamination. To avoid contaminating the gaskets, do not place the manifold face down on any surface.

6. Place the center of the manifold over the flow cell. The fanned-out tubes should point to the left, toward the removable strip tube holder.
7. Press to ensure that the manifold goes all the way down and is securely in place. Snap the white clamp down over the manifold and flow cell to hold them in place.
8. Place the fanned-out tubes into the tube strip.
9. Plug the clustered tubes into the output manifold.

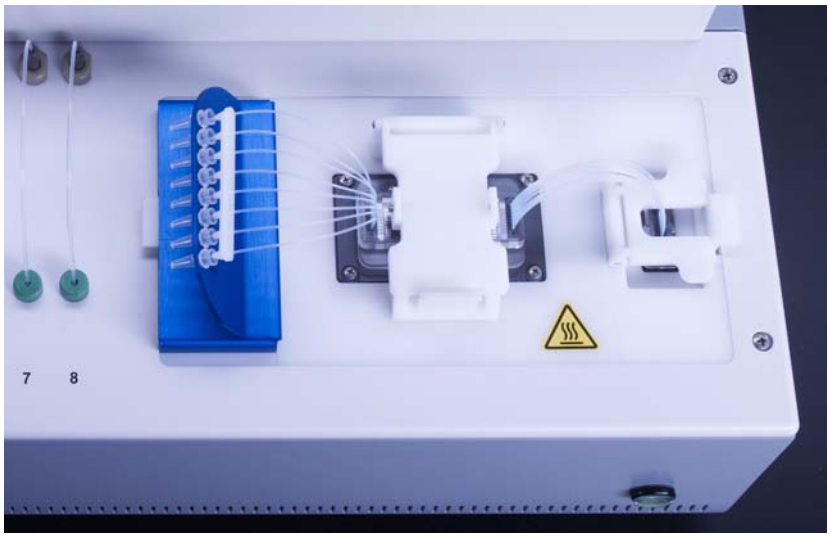

Figure 14 Flow Cell and Hybridization Manifold Installed

10. Start the appropriate recipe and check that the solution runs in all eight input tubes without obstruction. The tubes should all empty at the same rate.

11. If the tubes appear to be uneven, check to ensure that the flow cell is level. If the flow continues to be uneven, try replacing the strip tube.

Running a Recipe

For information about recipes, and instructions on how to stop and resume them, see Appendix C, *Recipes*.

1. In the Cluster Station software, select **File | Open Recipe**.
2. Open the desired recipe. For a list of recipes, see Table 3 on page 9.

   The protocol steps appear in the left sidebar. After the Cluster Station performs each step, a check mark appears beside it.

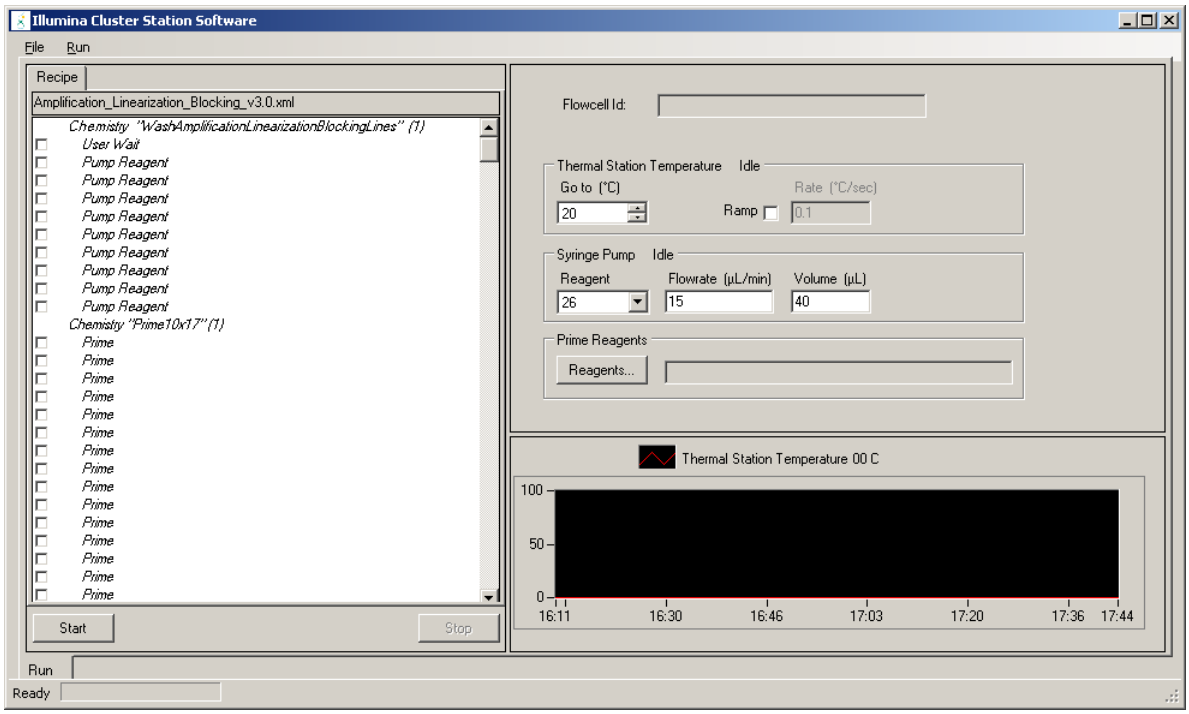

Figure 15 Amplification, Linearization, Blocking Recipe

3. Click **Start**.
4. If prompted, enter sample sheet data or navigate to an existing sample sheet, and then click **OK**.

   The prompt for a sample sheet is enabled in the configuration file RCM-Config.xml. Refer to *Configuring Sample Sheet Behavior* on page 100 for more information.

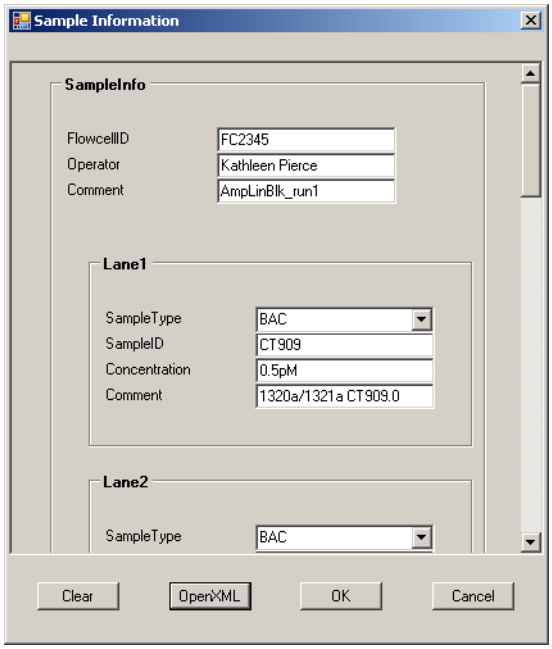

Figure 16 Sample Sheet Data

5. The system automatically generates a name for the run folder that will contain the data. Click **OK** to accept it, or enter a different name and click **OK**.

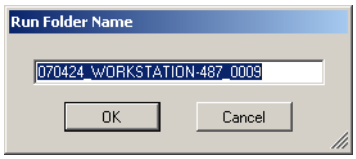

Figure 17 Run Folder

6. Follow the onscreen instructions to attach manifolds and load reagents. For manifold descriptions, see *Manifolds* on page 15. Follow the guidelines in *Loading Reagents* on page 20 to position the reagents.
7. When reagents are pumped through the eight lines of the Hybridization Manifold or through the eight lanes of the flow cell, check that the solution is pumped uniformly in *all* eight lines. If the solution does not flow evenly in some or all the lines, click **Stop** to pause the protocol, check the manifold connections, and then click **Resume** to restart the protocol.
8. When you connect the Amplification Manifold, check that each line is priming by observing the initial air gap flowing through the lines.

It is advisable to complete the full recipe without interruption. However, safe stopping points between recipes are clearly indicated. For more information, see *Safe Stopping Points During Cluster Generation* on page 26.

9. If prompted, continue to the wash protocol.

10. When the recipe finishes, select **File | Close Recipe**.
11. Do one of the following:
    - Proceed directly to the next recipe, if there is one.
    - After hybridizing the sequencing primer(s), sequence the flow cell within 4 hours.
    - Follow the directions in *Safe Stopping Points During Cluster Generation* on page 26 if you want to stop.

Safe Stopping Points During Cluster Generation

The variety of Cluster Station recipes give you great flexibility in planning your workflow. For more information, see *Protocol Times* on page 10 and *Scheduling* on page 11.

You can leave the flow cell in the Cluster Station for up to one day after the following recipes:

- Amplification_only_v<#>
- Amplification_Linearization_Blocking_v<#>
- Linearization_Blocking_only_v<#>

At the end of each of these recipes, the flow cell channels will be filled with Storage Buffer, making it safe to leave the flow cell on the Cluster Station overnight.

You can store the flow cell at 4°C for one month after the following recipes:

- Amplification_Linearization_Blocking_v<#>
- Linearization_Blocking_only_v<#>

You can store the flow cell at 4°C indefinitely after the following recipe:

- Amplification_only_v<#>

Follow the instructions in *Unloading the Flow Cell* on page 26 to unload the flow cell. Place the flow cell in a 50 ml conical tube filled with Storage Buffer and store it at 4°C.

After primer hybridization, the flow cell should be used for sequencing within 4 hours

Unloading the Flow Cell

1. Enter the following values in the Syringe Pump area to pump air into the inlet tubes of the manifold:

   Reagent: 26 (spare position with nothing in the reagent tube)

   Flowrate: 15 µl/minute

   Volume:
   - After amplification, with the hybridization manifold connected: 25 µl
   - After linearization, blocking, or primer hyb, with the amplification manifold connected: 40 µl

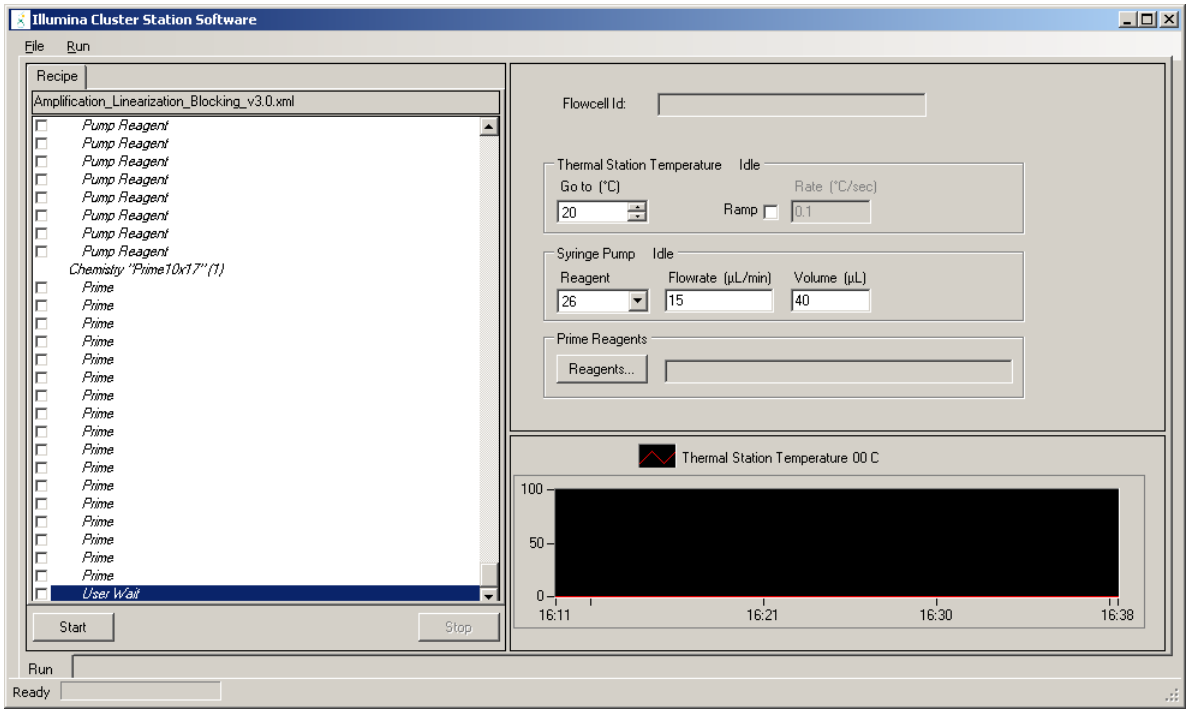

Figure 18 Setting Pump Controls to Unload Flow Cell

2. With your cursor in the **Volume** box, press **Enter**.

   This helps prevent fluid from the inlet tubes from spilling onto the flow cell when the manifold pressure is released.

3. Release the quick-connect clamp from the input manifold.
4. Lift the central white clamp off the manifold, but leave the output manifold clamp in place.
5. Remove the flow cell from the Cluster Station.
6. Release the quick-connect clamp from the output manifold and remove the manifold from the Cluster Station.
7. Using a lens cleaning tissue, gently wipe the flow cell stage and the metal posts on the input and output ports with water. Dry them thoroughly.

Washing the Lines

Perform a washing step before and after each run. There are three wash protocols:

- **WashAmponlyLines**—Washes the reagent lines that are used during isothermal amplification of clusters.
- **WashFullProtocolLines**—Washes all of the lines used during linearization, blocking, and primer hybridization.
- **DECON_Wash_Amplification_only_v<#>**—Performs a decontamination wash on the lines that are used during amplification. This wash should be run weekly for maintenance.

WashAmponlyLines

This protocol takes approximately 20 minutes. Run this protocol before and after amplification.

1. Disconnect the hybridization manifold.
2. Do one of the following:
   - When prompted at the end of the amplification recipe, agree to proceed to the washing protocol.
   - At any other time, select **File | Open Recipe** and select the WashAmpOnlyLines recipe. Click **Start**.
3. Connect the washing bridge to the input and output manifolds.
4. Place tubes filled with fresh water in reagent positions 1, 9, 10, 11, and 12. The exact volume is not important.
5. Click **OK** to start the wash.
6. Check for proper flow in each line of the washing bridge. Air movement in the line is a sign of good flow. You can introduce an air bubble to test the flow by taking one of the reagent lines out of water as it is being pumped.

   At the end of the wash protocol, a message alerts you that the wash is finished.
7. Click **OK**.

WashFullProtocolLines

This protocol takes approximately 21 minutes to run. Run this protocol before linearization and after primer hybridization.

1. Disconnect the Amplification or Hybridization Manifold from the input and output manifolds.
2. Do one of the following:
   - When prompted at the end of a recipe, agree to proceed to the washing protocol.
   - At any other time, select **File | Open Recipe** and select the WashFullProtocolLines recipe. Click **Start**.
3. Connect the washing bridge to the input and output manifolds.
4. Place tubes filled with clean water in reagent positions 9, 10, 11, 12, 14, 15, 17, 18, 1, 3, 5 and 7. The exact volume is not important.
5. Click **OK** to start the wash.
6. Check for proper flow in each line of the washing bridge. Air movement in the line is a sign of good flow. You can introduce an air bubble to test the flow by taking one of the reagent lines out of water as it is being pumped.

   At the end of the wash protocol, a message alerts you that the wash is finished.
7. Click **OK**.

8. If you plan to continue a protocol, connect the appropriate manifold to the flow cell and to the input and output manifolds.

Weekly Maintenance Wash

This protocol takes approximately 21 minutes to run. Perform a maintenance wash once a week to help prevent blockages and microbial growth in the fluidics system.

1. If an amplification or hybridization manifold is connected to the input and output manifolds, disconnect it.
2. From the Cluster Station software, select **File | Open Recipe**. Select the recipe called DECON_Wash_Amplification_only_v<#>.
3. Click **Start**.
4. Following the on-screen instructions, connect the washing bridge to the input and output manifolds.
5. Place 50 ml tubes containing approximately 25 ml of 5% DECON in positions 1, 9, 10, 11, and 12. Click **OK**.
6. Following the on-screen instructions, replace the 5% DECON tubes with fresh 50 ml tubes containing approximately 40 ml of clean water. Click **OK**.
7. Following the on-screen instructions, replace the water in the 50 ml tubes with another 40 ml of clean water. Click **OK**.

Troubleshooting

The controls in the Manual Controls/Setup window in the Cluster Station software are primarily used by Field Service for configuration or troubleshooting.

Setting the Thermal Station Temperature

The Thermal Station Temperature panel allows you to manually control the temperature of the thermal station.

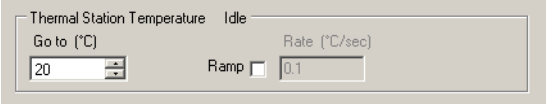

Figure 19 Thermal Station Temperature

1. Enter the desired temperature (°C) in the **Go To** box.
2. If you want to ramp the temperature at a certain rate, select the **Ramp** checkbox.
3. In the **Rate** box, enter the rate at which the temperature should increase (positive number) or decrease (negative number).

The recommended rate for temperature change is 1 degree per second.

4. Press **Enter**.

Pumping Reagents

The Syringe Pump manual control pumps reagents through the flow cell or washing bridge.

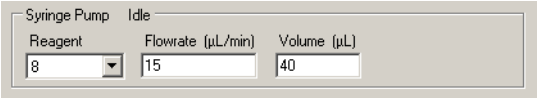

Figure 20 Syringe Pump

1. In the **Syringe Pump** box, select the reagent position from which to pump.

   Reagents 1 through 18 correspond to the reagent positions illustrated.

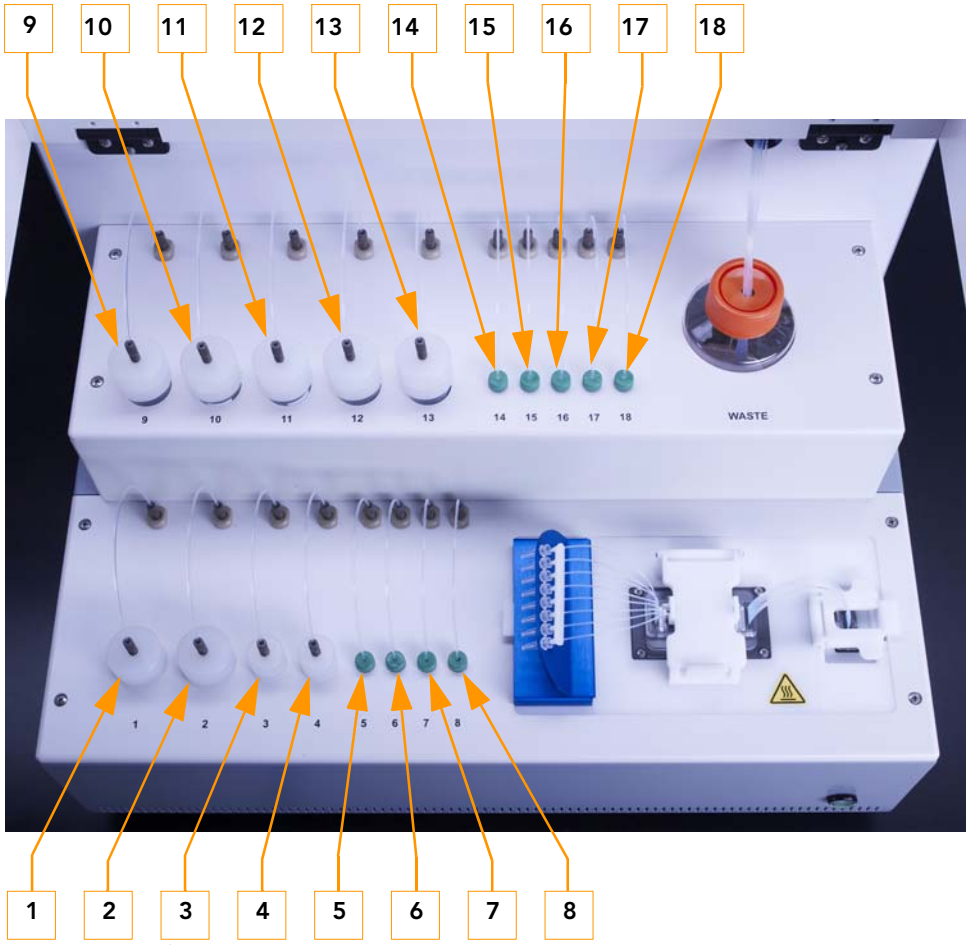

Figure 21 Cluster Station Reagent Positions

Positions 19 through 26 are not connected to any reagent lines and can be used to pump air into the system.

2. In the **Flowrate** box, type in the rate at which the reagents should be pumped.

NOTE

The recommended flow rate for pumping through the washing bridge is 240 µl/min.
The recommended rates for pumping through flow cells are 15 µl/min or 60 µl/min.

3. In the **Volume** box, type in the volume (µl) to be pumped.
4. With the cursor in the **Volume** box, press **Enter**.

Priming Reagents to Waste

The Prime Reagents manual control allows you to prime reagents directly to waste, bypassing the flow cell.

1. In the Prime Reagents panel, click **Reagents**.

   The Select Reagents dialog box appears.

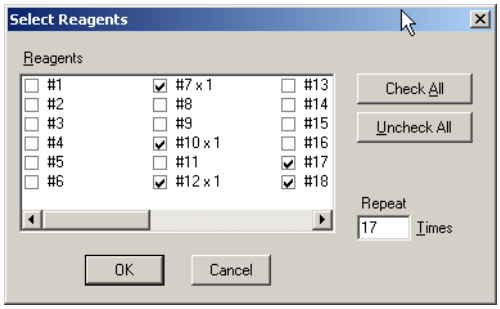

Figure 22 Select Reagents

2. In the **Repeat** box, enter the number of times to prime the lines. Each firing of the priming pump pumps 20 µl of fluid.

   For reagent positions 5 through 8, 13 primes are needed to fill the reagent lines up to the 26-way valve inside the Cluster Station.

   For all other reagent positions, 17 primes are needed to fill the reagent lines.

3. Select the checkbox beside each reagent position that you want to prime. Click **Check All** if you want to prime all reagent positions.

4. If you click **Check All**, clear the checkboxes beside positions 19–26. These ports are currently not connected to any reagent lines.

5. Click **OK**.

6. Press **Enter**.

   The Prime Reagents panel lists all the positions it is priming.

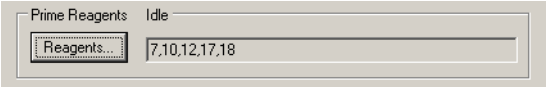

Figure 23 Lines Primed

Temperature Profile

The Temperature Profile window shows the temperature from the start of the run to the time the protocol stops.

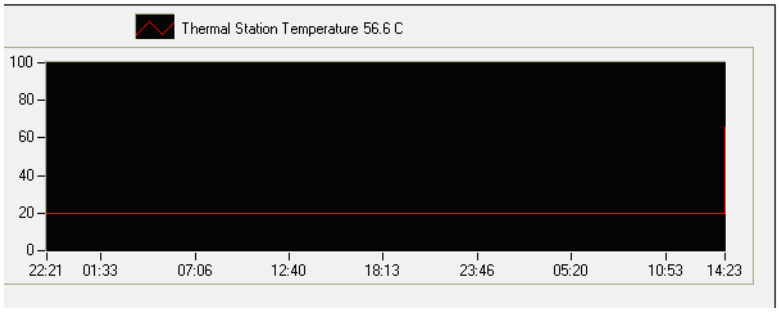

Figure 24 Temperature Profile

Software Errors

Selector Valve Error

If the Cluster Station power is turned off while a protocol is running, then you might see the following error message when you restart the instrument:

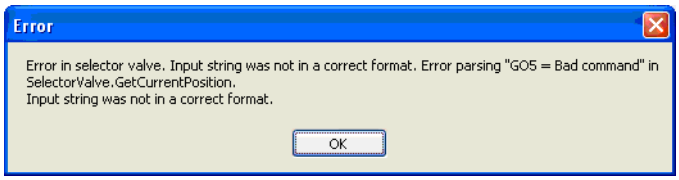

Figure 25 Selector Valve Error Message

To fix the problem:

1. Open the <install directory>\bin\release folder and double click HCMConsole.exe.
2. Type `pumpinit` at the command prompt.

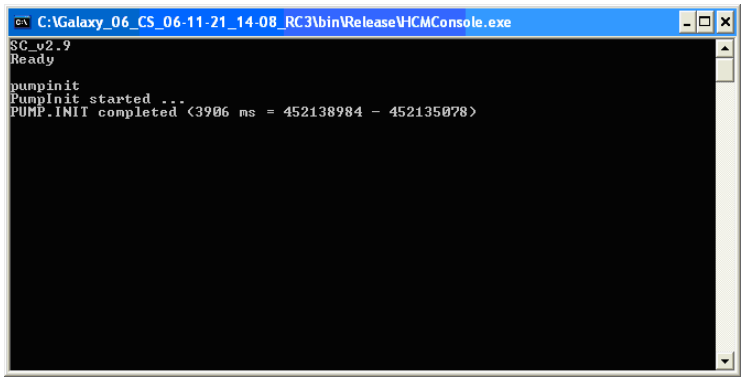

Figure 26 Pumpinit Command

3. Wait for the initialization to finish, and then close the command window.
4. Start the Cluster Station software.
5. Open a recipe and click **Start**.
6. If the message appears again, turn the Cluster Station and PC off and then on again. Repeat the pumpinit command.
7. If the message appears yet again, contact Illumina Technical Support.

FlowcellTmpr Error

You might see the FlowcellTmpr Error message if any of the following occur:

- You open RCM.exe when the Cluster Station is turned off or before establishing communication with Perle/Edgeport USB/COM box
- You open RCM.exe while the HCMConsole.exe is running
- You accidentally launch two instances of RCM.exe. It is possible for the software to take up to 2 minutes to launch.

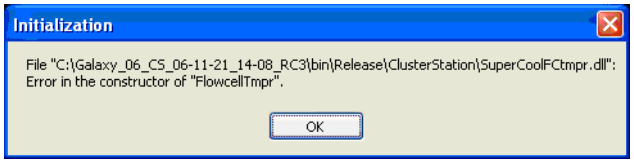

Figure 27 Flowcell Tmpr Error Message

To fix the problem:

1. Turn the Cluster Station on and launch RCM.exe again.
2. If the error persists, take the following actions:
   a. Right click **My Computer** and select **Manage**.
   b. Expand the System Tools list and click **Device Manager**.
   c. Expand the Ports (COM & LPT) entry and check to see if COM ports have been assigned to the Perle/Edgeport box. If no COM ports are assigned, then only COM1 will appear.

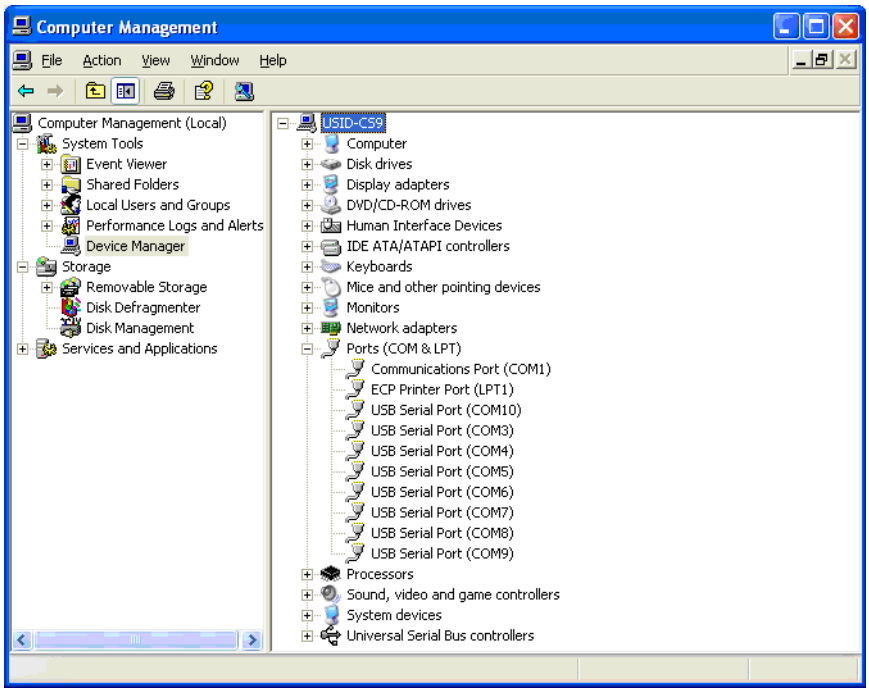

Figure 28 COM Port Settings in Device Manager

   d. Check the USB cable connections.
   e. Turn the Cluster Station and PC off and then on again, and recheck the COM ports.
   f. Launch RCM.exe again.
3. If the message appears again, contact Illumina Technical Support.

Chapter 3

Using the Genome Analyzer

Topics

Introduction

The Genome Analyzer sequences clustered template DNA using a robust four-color DNA Sequencing-By-Synthesis (SBS) technology that employs reversible terminators with removable fluorescence. This approach provides a high degree of sequencing accuracy even through homopolymeric regions.

High sensitivity fluorescence detection is achieved using laser excitation and total internal reflection optics. Short sequence reads are aligned against a reference genome and genetic differences are called using a specially developed data pipeline.

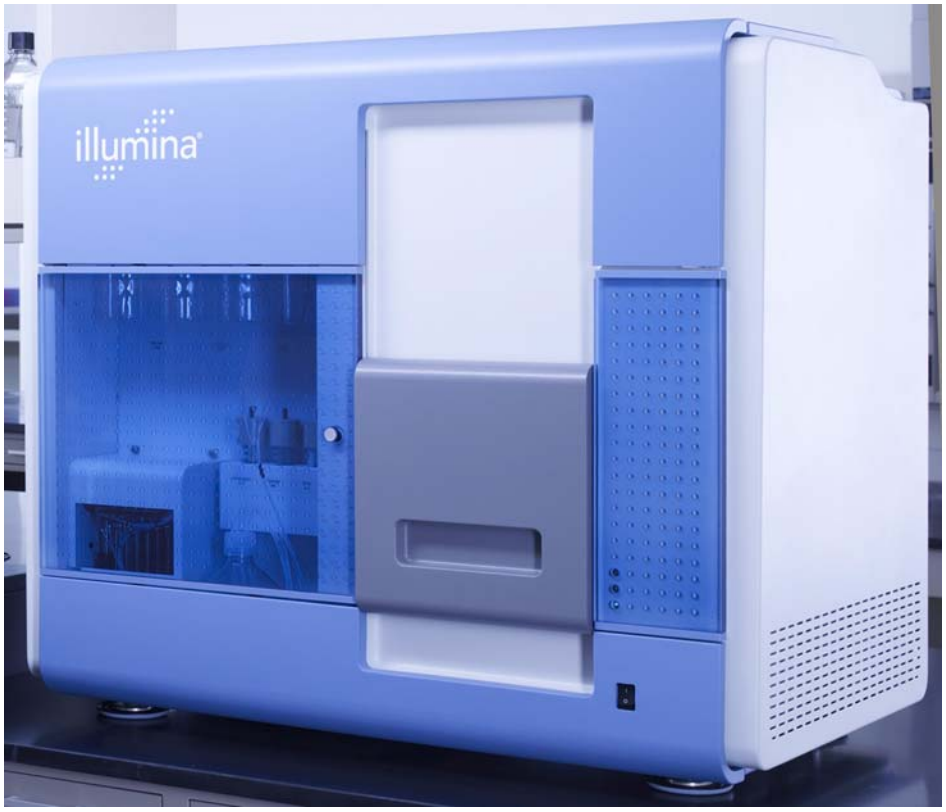

Figure 29 Genome Analyzer

Workflow

To perform a sequencing run, follow all of these procedures in the order shown.

1. *Performing a Pre-Run Wash* on page 49
2. *Preparing, Loading, & Priming Reagents* on page 50
3. *Cleaning and Installing the Prism* on page 53
4. *Cleaning and Installing the Flow Cell* on page 55

5. *Checking for Leaks and Proper Reagent Delivery* on page 58
6. *Performing First-Base Incorporation* on page 60
7. *Loading the Flow Cell with Scan Buffer* on page 61
8. *Applying Oil* on page 59
9. *Adjusting Focus* on page 62
10. *Checking Quality Metrics* on page 69
11. *Completing the Run* on page 71
12. *Performing Post-Run Procedures* on page 71
13. *Updating the Offsets File* on page 74

Components

All operator activity on the instrument occurs in two main compartments:

- Reagent Compartment
- Imaging Compartment

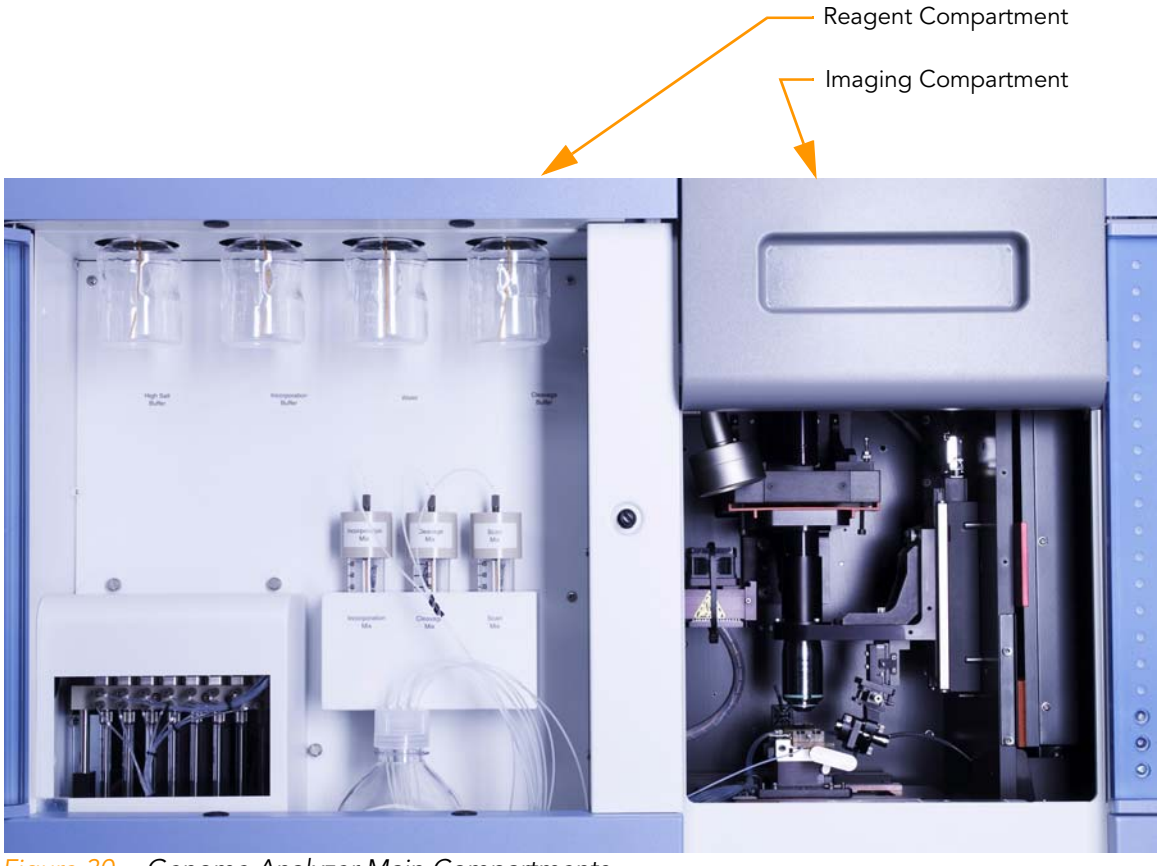

Figure 30 Genome Analyzer Main Compartments

Reagent Compartment

The left-side reagent compartment holds active reagents, buffers, wash solutions, and the liquid waste container. The waste bottle receives liquid waste from the Kloehn Pump.

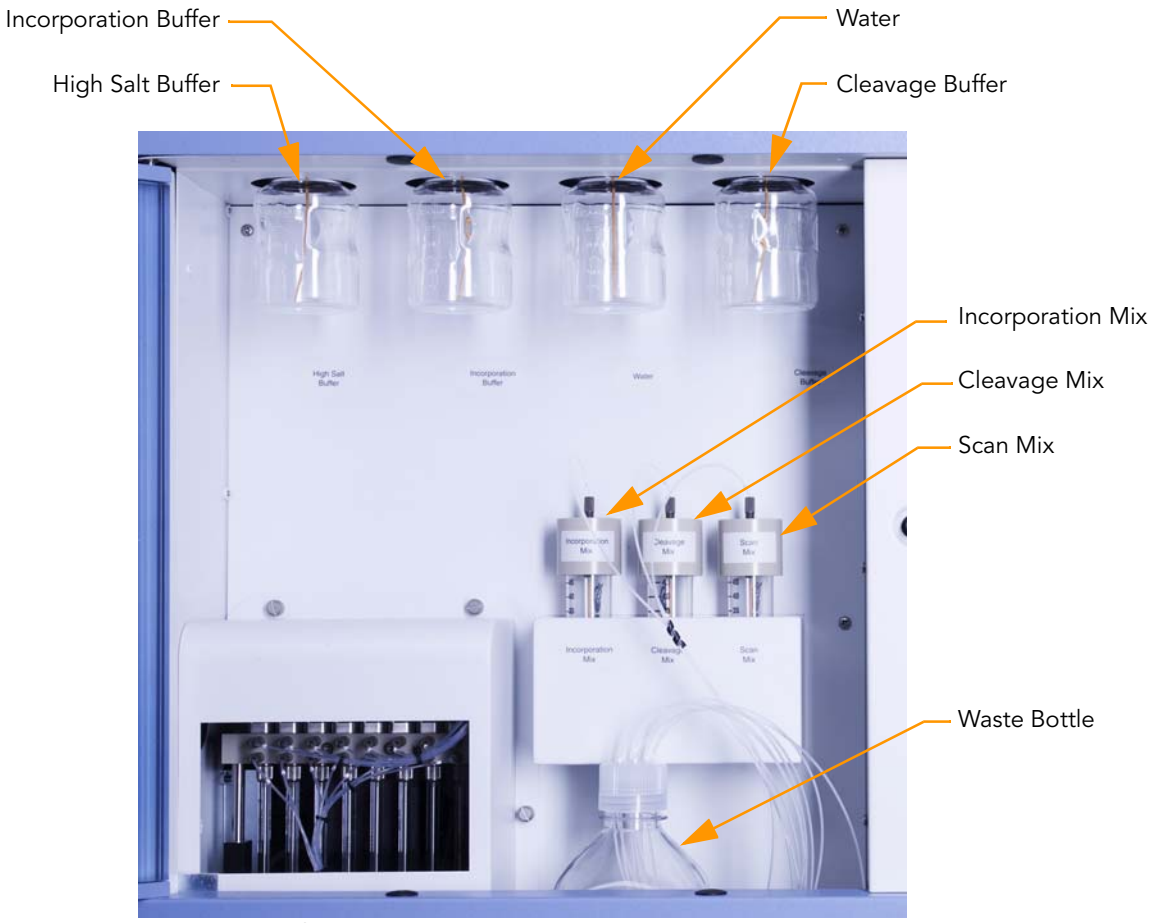

Figure 31 *Genome Analyzer Reagent Compartment*

Each reagent is numbered according to the position it connects to on the fluidic valve. The 250 ml reagent bottles are connected to threaded bottle receptacles on the instrument. The 50 ml tube caps are threaded onto reagent mix tubes before placing the capped tube into the instrument cooler.

Figure 32 *Reagent Positions*

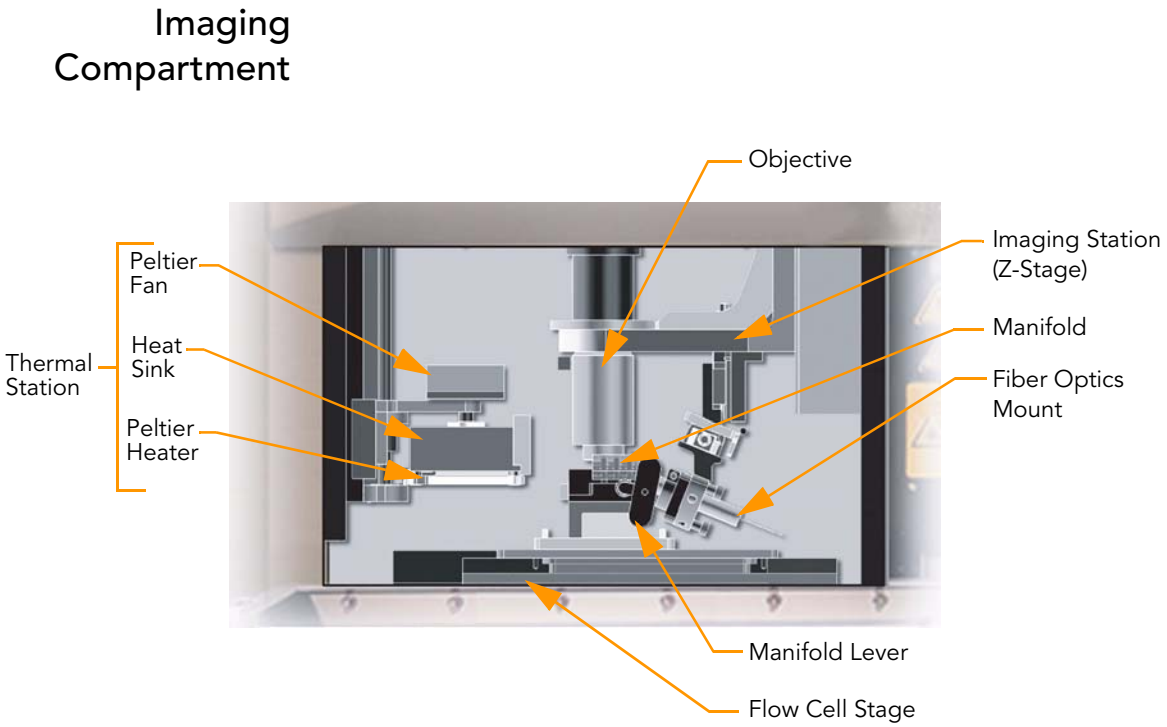

Figure 33 Genome Analyzer Imaging Compartment

The flow cell stage moves along the X (left-right) and Y (front-back) axes.

The Thermal Station and the Imaging Station (Z-Stage) move along the Z axis.

The flow cell is clamped under front and rear plumbing manifolds onto a stage that moves between Thermal and Imaging stations.

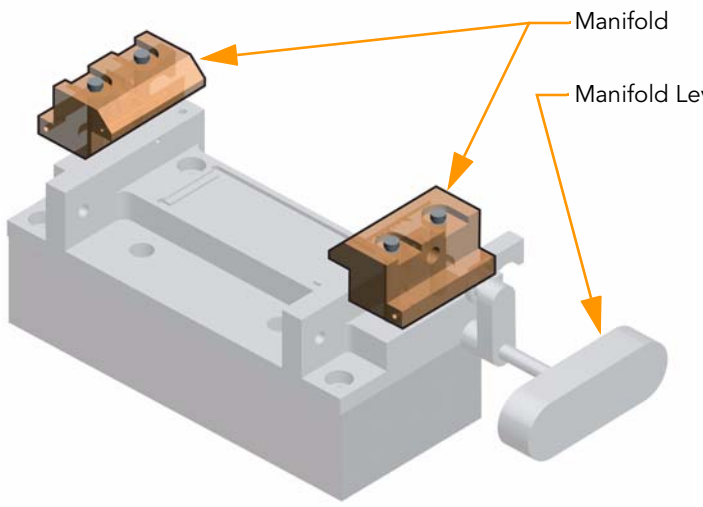

Figure 34 Front and Rear Plumbing Manifolds

Starting the Genome Analyzer

It is best to leave the Genome Analyzer on at all times. Turn it off only if it will remain idle for more than three days. However, you should restart the computer before each run to ensure that the software is properly initialized.

1. Turn the main switch to the ON position.

   After a short delay the instrument emits a regular buzzing sound from the top-right-rear region of the chassis. The sound is a normal by-product of a stable instrument initialization.

2. Restart the computer and log on to the operating system.

   The default logon is:

   Username: sbsuser

   Password: sbs123

   If the default values do not work, consult your IT personnel to find out the correct user name and password for your site.

3. Delete the data from all previous runs to ensure adequate disk space (approximately 1 TB per run). If deleting the large files is excessively time consuming, perform a quick reformat of the data drive, as follows:
   a. Right-click **My Computer** and select **Manage**.
   b. In the tree on the left side of the screen, select **Storage | Disk Management**.
   c. Right-click **D Partition** and select **Format**.
   d. Select the **Perform a Quick Format** checkbox. Leave all other parameters at the default values
   e. Click **OK**.

4. Create a new SbsData folder on the D partition to hold the run data. For more information, see Appendix A, *Run Folders*.

5. Double-click the Illumina Genome Analyzer Data Collection Software icon on the desktop.

6. The home window for the Genome Analyzer software appears.

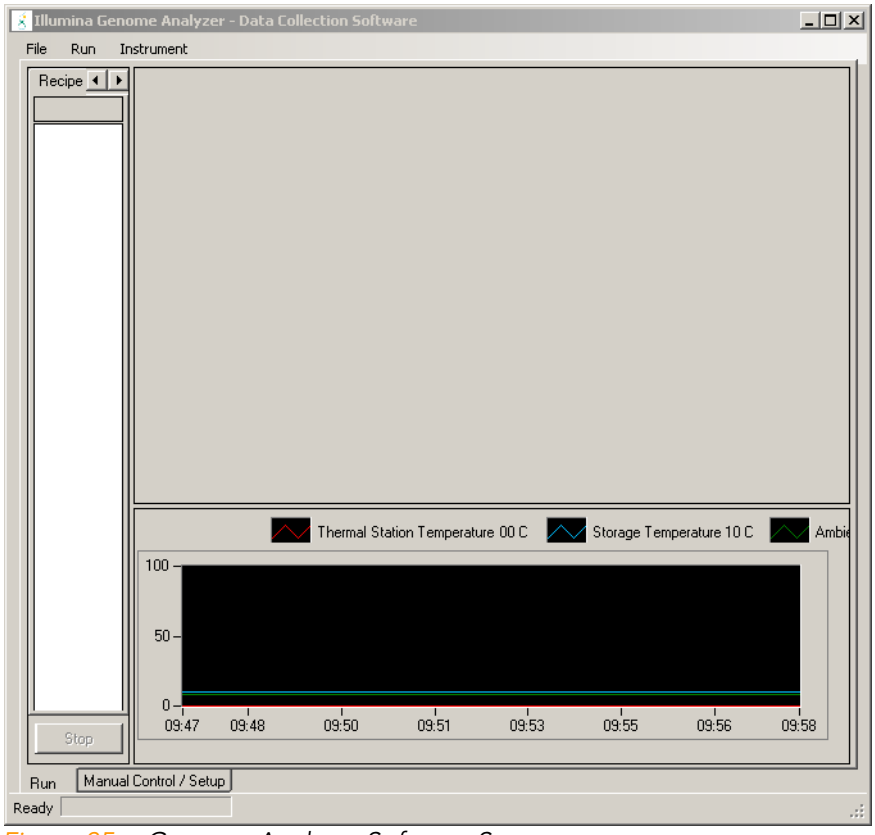

Figure 35 Genome Analyzer Software Screen

7. Click the Manual Control/Setup tab. Perform any operation (e.g., click **Take a Picture**) to trigger the Genome Analyzer initialization. A green bar at the bottom-left of the screen shows the progress of the initialization routine.

NOTE

The software will ignore inputs until the routine is complete.

Software User Interface

This section describes the Genome Analyzer software interface.

Run and Manual Control/Setup Windows

The Genome Analyzer software has two main windows, the Run window and the Manual Control/Setup window. Menu commands that are available in one window may not be available in the other.

The software opens to the Run window.

The tabs for toggling between the Run window and the Manual Control window are in the bottom-left corner of the screen.

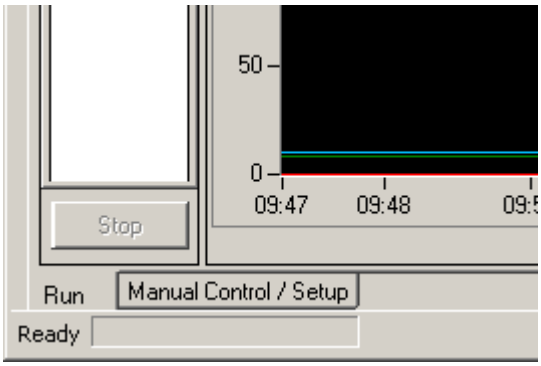

Figure 36 Run and Manual Control/Setup Windows

Recipe and Image Cycle Tabs

Two tab views are available in the left column of the Run window. By default, the Recipe tab is in view.

The Recipe tab lets you control and monitor recipes.

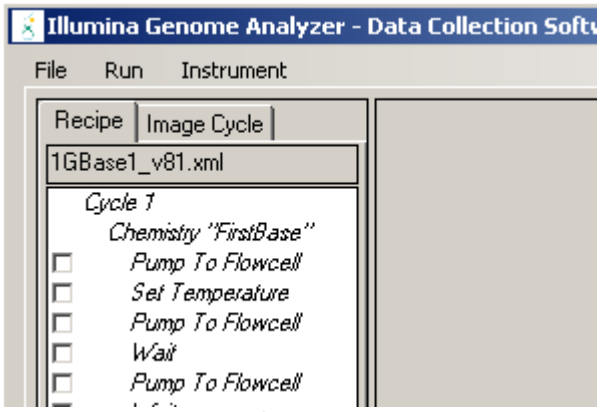

Figure 37 Recipe Tab

The Image Cycle tab lets you view the progress of the scanning run. The left column shows the entire flow cell, using color codes for each tile:

- Blue tiles have been imaged.
- White tiles are queued for imaging.
- Grey tiles are not defined in the current run.

The right side of the window shows the photographs being taken of the current tile.

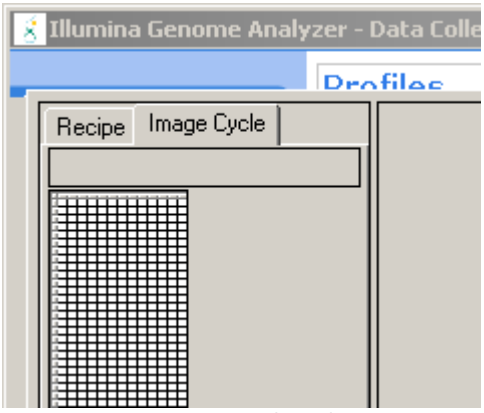

Figure 38 Image Cycle Tab

Image Controls

The Genome Analyzer software provides various tools for adjusting the image and discovering information.

The image display uses 8 bits of data to display color, while the TIFF files that are saved contain 16-bit color images. The Show False Color and Auto Scale options provide methods for simulating the larger dynamic range on the computer monitor.

Table 6 Genome Analyzer Image Controls

Adjustment	Action
Zoom in	Click and drag a rectangle over the area you want to see.
Move the zoomed image	Right-click and select **Center Here**. The display adjusts so that the area you clicked on is at the center.
Zoom out	Right-click over the image and select **Zoom Out** to zoom out one level. Right-click over the image and select **Zoom Out All** to zoom out all the way.
Modify the color display	Right-click over the image and select **Show | Color** or **Show | False Color**. Show Color displays the laser light colors used to acquire the image: blue for the focus tracking images, and red (A/C) or green (G/T) for the intensity values. Show False Color lets you customize the color contrast so that it is easier to see peak intensities. Select from: • None • Blue-Green • Green-Blue • Blue-Red • Red-Blue • Red-Green • Green-Red When you select a two-color option, the Cluster Station uses the upper 8 bits of the Tiff 16-color range for one color (e.g., blue), and the lower 8 bits for the other color (e.g., green).
View or hide the center mark	Right-click over the image and select **Show | Center Mark** to toggle the display of the center mark, which indicates the stage's current X and Y values.

Table 6 Genome Analyzer Image Controls

Adjustment	Action
See intensity values	Roll the mouse over a point of raised intensity to display its pixel position and intensity value.
Scale the intensity values	Right-click over the image and select **Auto Scale | On** or **Auto Scale | Off**. When auto scaling is on, the minimum intensity value is mapped to 0 and the maximum intensity value is mapped to 255. When auto scaling is off, the system maps the 16-bit range to an intensity range that you define in RCMConfig.xml. All data intensity values at or below the minimum are mapped to 0 and all values at or above the maximum are mapped to 255. Generally, this mapping is a linear function.
See focus quality and uniformity	Hover the mouse over the whole flow cell in the left column to display the focal quality and uniformity. Note: This feature currently does not support high-density clusters (> 15K).
Save the image	Right-click on the image and select **Save As** to save the image as a TIFF file.

Pump Control

Some setup and maintenance protocols require you to set the pump control parameters.

Figure 39 Pump Control Area

Table 7 Pump Controls

Control	Description
Command	**Pump**—Transfers liquid from the port indicated in the **Solution** box to the location indicated in the **To** box. This command is used in many procedures, such as washes.
To	**Flow Cell**—Directs the liquid through the flow cell. The Waste option is not used.
Solution	[1–8]—The port from which the instrument will draw liquid. Note: Solution 8 is currently air, rather than a liquid. You will select this position when you remove flow cells from the system, so that fluid does not siphon down the lines.
Volume	The volume to be transferred in a single pump event, in microliters.
Aspiration rate	The rate at which liquid is removed from the source, in microliters/minute.
Dispense rate	The rate at which liquid is placed into the target location, in microliters/minute.

Basic Procedures

Washing the Lines

It is important to regularly wash the lines of the Genome Analyzer. There are several types of wash:

- *Performing a Pre-Run Wash* on page 49
- *Storage and Monthly Maintenance Wash* on page 45 (including a *1 ml Water Wash* and *1 ml 1N NaOH Wash*)
- *Post-Run Wash* on page 71

Storage and Monthly Maintenance Wash

This wash has three parts: the 1 ml water wash, the 1 ml NaOH wash, and the storage wash. Only perform the storage wash if you plan to store the Genome Analyzer for more than three days.

Perform this wash once monthly for maintenance, and any time you plan to leave the Genome Analyzer to sit idle for more than three days. The monthly wash uses the same instrument cycle as the pre-run wash, but uses a stronger base (1N NaOH) and filtered cleaning liquids.

Consumables

User-Supplied

Item	Description
Lens cleaning tissue	
Nylon filter	0.2 µm pore size
Deionized water	0.5 L, filtered with a 0.2 µm nylon filter
1N NaOH	0.5 L, filtered with a 0.2 µm nylon filter

Illumina-Supplied

Item	Description
Instrument Wash Solution	Three bottles and three tubes prefilled with optimized instrument washing solution

1 ml Water Wash

If you washed the instrument after its most recent run, skip the 1 ml water wash and begin with the 1 ml NaOH wash.

If the instrument has just completed a run, and has not been washed, proceed as follows:

1. Click the Manual Control/Setup tab.
2. Click **Load Flow Cell**. You can use the flow cell from the last run for the cleaning.
3. Do one of the following:
   - Load a clean, dry flow cell.
   - If the flow cell is still in the instrument compartment from the last run, remove it. Clean the used flow cell with deionized water, and then dry it with a lens-cleaning tissue. Reload the flow cell into the Genome Analyzer.
4. Load the instrument with prefiltered water as follows:

   25 ml for positions 1, 6, and 3

   50 ml for port positions 4, 5, and 7

Rotate the tubes while holding the caps stationary, to prevent crimps and twisting in the liquid delivery lines.

   Port position 2 is already loaded with water.

5. Check for leaks as follows:
   a. Wipe the line where the flow cell touches the manifold with a lens-cleaning tissue to check for leaks.

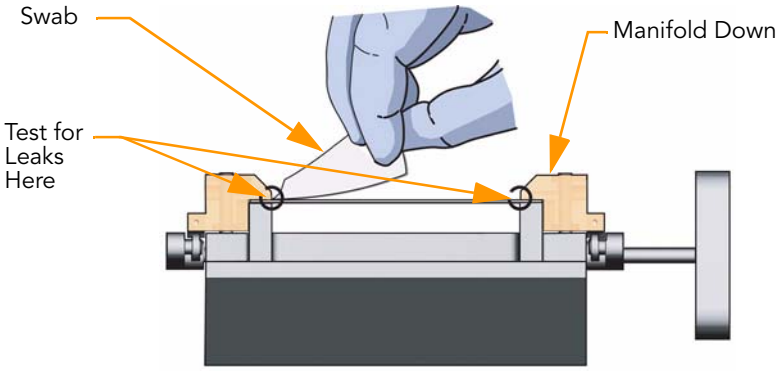

Figure 40 Testing for Leaks

   b. Click the Manual Control/Setup tab.
   c. In the Pump area, set the parameters as follows:
      Command: Pump to Flow Cell
      To: Flowcell
      Solution: 5
      Volume (µl): 200
      Aspiration Rate (µl/minute): 60
      Dispense Rate (µl/minute): 2000
6. With the cursor in the **Dispense Rate** box, press **Enter**.
7. Wait until the air bubbles disappear from the lanes of the flow cell. If they remain, refer to *Checking for Leaks and Proper Reagent Delivery* on page 58 for solutions.

8. Click the Run tab.
9. Select **File | Open Recipe**.
10. Open the PreWash_v<#>.xml recipe file.
11. Click **Start** and enter a file name.

    The wash cycle runs for approximately 20 minutes.

1 ml 1N NaOH Wash

1. Load the instrument with 1N NaOH as follows:

   25 ml for positions 1, 6, and 3

   50 ml for port positions 4, 5, 2, and 7

Rotate the tubes while holding the caps stationary, to prevent crimps and twisting in the liquid delivery lines.

2. If you just performed the 1 ml Water Wash, leave the flow cell in place. Otherwise, load a used flow cell as follows:
   a. Click the Manual Control/Setup tab.
   b. Click **Load Flow Cell**.
   c. Clean a used flow cell with deionized water, and then dry it with a lens-cleaning tissue.
   d. Load the clean, dry flow cell.
3. Click the Run tab.
4. Select **File | Open Recipe**.
5. Open the PostWash_v<#>.xml recipe file.
6. Click **Start** and enter a file name.

   The wash cycle runs for approximately 45 minutes.

This concludes the monthly wash cycle.

Storage Wash

If you plan to leave the Genome Analyzer idle for more than three days, perform this wash after the water wash and 1N NaOH wash.

1. Load wash solutions into port positions 1, 3, 4, 5, 6, and 7.

   Position 2 remains loaded with water.
2. Remove any tubing connected to port position 8 and close the port with the appropriate stopper.
3. Click the Run tab.
4. Select **File | Open Recipe**.
5. Open the PostWash_v<#>.xml wash recipe file.
6. Click **Start**.
7. When the run finishes, click the Manual Control tab.

8. In the Pump area, set the parameters as follows:
   Solution: 8
   Volume: 0
9. With the cursor in the **Volume** box, press **Enter**.
10. Leave the flow cell in the instrument to prevent siphoning.
11. Close the Genome Analyzer software and shut down the computer.
12. Turn the Genome Analyzer power switch to the OFF position.

Resuming Use after Short- or Long-Term Storage

1. Turn on the Genome Analyzer.
2. Start the computer and log on to the operating system.
3. Open the Genome Analyzer software.
4. Load wash solutions into port positions 1, 3, 4, 5, 6, and 7.
5. Load 0.5 L filtered, deionized water into Position 2.
6. Click the Run tab.
7. Select **File | Open Recipe**.
8. Open the PreWash_v<#>.xml wash recipe.
9. Click **Start**.

Unloading a Flow Cell from the Stage

1. Click **Load Flowcell** to slide the stage forward.
2. Select **Instrument | Unlock Door** to release the door to the imaging compartment.
3. Click the Manual Control/Setup tab.
4. In the Pump area, set the following values:
   Command: Pump
   To: Flowcell
   Solution: 8 (to prevent siphoning reagents)
   Volume: 0
   Aspiration Rate: 60
   Dispense Rate: 2000
5. With the cursor in the **Dispense Rate** box, press **Enter**.
6. Turn the manifold handle clockwise to lift the manifolds.

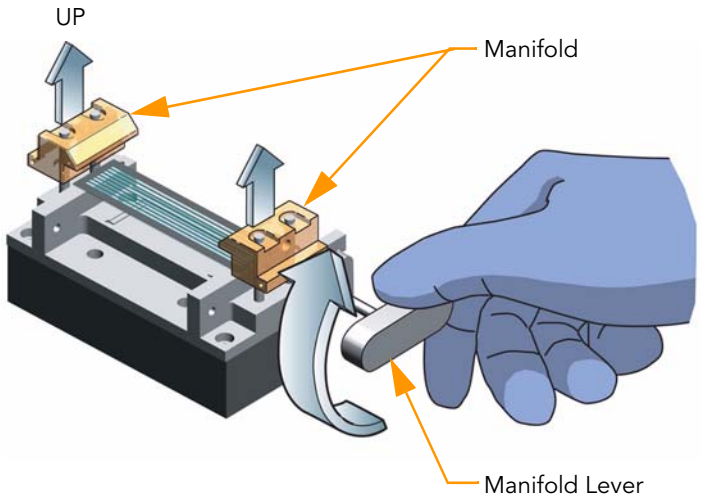

Figure 41 Lifting Front and Rear Manifolds

7. Slide the flow cell to the left to clear the manifolds, and then lift it out of the instrument.

Performing a Pre-Run Wash

You must perform a pre-run wash if the instrument has been idle for a day or more, and before changing and priming reagents. The wash flushes 1 ml of instrument wash reagents through each reagent port and out to a waste container. Run time is approximately 20 minutes.

After the wash, check the total volume in the waste container closely to confirm the stability of the reagent delivery system. The primary indicators of a stable fluid delivery system are (1) air-free volumes in syringe barrel and flow cell and (2) expected delivery volumes from the wash cycle. Both are necessary for optimal sequencing performance.

Consumables

User-Supplied

- Lens cleaning tissue
- Nylon filter (0.2 µm pore size)
- Deionized water for washes (0.5 L, filtered with a 0.2 µm nylon filter)

Illumina-Supplied

- Wash Solution

1. Load the instrument with a used flow cell. Refer to *Loading the Flow Cell* on page 56.
2. Load the instrument with solutions as follows:
   - 50 ml Instrument Wash solution for port positions 1, 6, 3, 4, 5, and 7
   - 250 ml deionized water for port position 2

Rotate the tubes while holding the caps stationary, to prevent crimps and twisting in the liquid delivery lines.

3. Loosen and remove the waste tubing.
4. Bundle all waste tubes with parafilm, making sure to keep all of the ends even.
5. Place the bundled tube ends into a 15 ml tube.
6. Click the Run tab.
7. Select **File | Open Recipe**.
8. Open the PreWash_v<#>.xml recipe file.
9. Click **Start**.

   The wash cycle runs for approximately 20 minutes.

   Reagents are delivered 1 ml at a time. You should collect a total volume of 7 ml.
10. Record the delivery volume in the lab tracking worksheet. If the measured volume is less than 90% of the expected value, do the following:
    a. Check for leaks.
    b. Repeat the wash cycle.
    c. Collect and measure each 1 ml delivery.
11. Proceed to *Preparing, Loading, & Priming Reagents* on page 50.

Preparing, Loading, & Priming Reagents

Preparing Reagents

For instructions on preparing reagents, refer to the *Preparing Reagents for the* Genome Analyzer document that you received with the documentation. Prepare all the reagents as described before proceeding.

Loading Reagents

Reagents loaded onto the Genome Analyzer must be used in a sequencing run the same day.

Safe Handling Conventions

To prevent cross-contamination of reagents, especially the Incorporation and Cleavage mixes, establish safe handling conventions such as:

- Always remove and replace one bottle or tube at a time.
- Always install the Cleavage Reagent *last* to avoid cross-contamination.
- Keep the Scan Buffer, dNTP mix and Cleavage Mix on ice until you load them into the instrument.

- Invert all Wash solutions to mix them before loading them into the instrument.

Reagent Positions

Load the prepared reagents into the appropriate position on the Genome Analyzer, as shown in this image and table. When you attach the 50 ml tubes, hold the caps stationary and rotate the tubes to prevent crimps in the liquid delivery lines.

Figure 42 Reagent Positions

Table 8 Genome Analyzer Reagent Positions

Solution #	Size	Contents
1	50 ml Tube	Incorporation Mix
2	250 ml Bottle	Deionized Water
3	50 ml Tube	Scan Mix
4	250 ml Bottle	High Salt Buffer
5	250 ml Bottle	Incorporation Buffer
6	50 ml Tube	Cleavage Mix
7	250 ml Bottle	Cleavage Buffer

Priming Reagents

Priming volumes are a key indicator of a stable fluid delivery system. The measured volumes must be within 10% of normal for optimal sequencing performance.

Before each run, you must prime all of the plumbing lines with the reagents. You will collect a set of liquid deliveries through all valve ports out to a waste volume, and then check total volume to confirm the stability of the reagent delivery system.

Consumables

Illumina-Supplied

Obtain one of the following:

Part #	Item
1002427	SBS 18-Cycle Kit
1001599	SBS 26-Cycle Kit
1001461	SBS 36-Cycle Kit

1. Loosen and remove the waste tubing from the waste bottle.
2. Bundle all waste tubes so that the ends are even with each other, and wrap them with parafilm.
3. Place the bundled tube ends into a small vial (approximately 15 ml or a 50 ml conical tube).
4. Click the Run tab in the Data Collection software window.
5. Select **File | Open Recipe**.
6. Open the Prime_v<#>.xml recipe file.
7. Click **Start**.
8. Collect all of the waste from the priming recipe and ensure that the volume is 6.4 ml.
9. Record the delivery volume in the lab tracking worksheet. If the measured volume differs from the expected value by more than 10%, repeat the priming procedure.

   If the delivered volume still differs from the expected volume by more than 10%, take the following steps:
10. Click the Manual Control/Setup tab.
    a. In the Pump area, set the following values:
       Command: Pump
       To: Flowcell
       Solution: 8
       Volume: 0
       Aspiration Rate: 60
       Dispense Rate: 2000
    b. Click **Load Flow Cell**.
    c. Lift the manifolds and reposition the flow cell.
    d. Repeat the priming procedure.
11. Proceed to *Cleaning and Installing the Prism* on page 53.

Cleaning and Installing the Prism

The prism sits under the flow cell and serves a critical optical function.

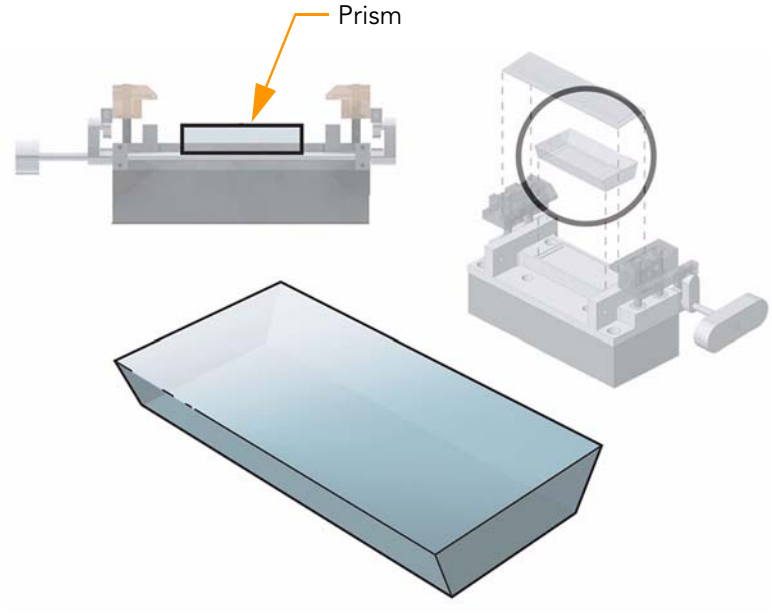

Figure 43 Prism

Handling the Prism

Always wear powder-free latex gloves when handling the prism. Hold it by the top front and rear edges, or by all four corners if using two hands.

CAUTION

Exercise extreme care when handling the prism to prevent chipping, as this might degrade its optical function. The critical surface is the right side, which is the surface of laser entry.

Cleaning the Prism

Consumables

User-Supplied

- Lens cleaning tissue
- 100% ethanol or Spectrophotometer-grade methanol

1. Put on new gloves.
2. Place the prism on a fresh lens cleaning tissue on the benchtop.
3. Remove the oil by gently washing the prism with a stream of ethanol.
4. Fold a lens cleaning tissue to approximately the size of the prism. Wet the edge of the tissue with ethanol and wipe off the surface with a single sweeping motion. Repeat, refolding the tissue with each wipe, until the prism is completely clean.

5. To tell if the prism is clean, observe it under direct light from a variety of angles. Pay particular attention to the surface that will face the laser entry (right-hand side when sitting in the holder) and the large top surface.
6. Protect the prism from dust until you place it into the Genome Analyzer.

Installing the Prism

Install the prism before installing the flow cell.

1. Fold a lens cleaning tissue and wet it with ethanol.
2. Wipe the recessed surface of the prism holder to remove oil that may have been spilled during the previous run.
3. Look at the prism under direct light to identify the slanted surface that has the highest optical quality: i.e., clean and free of chips or scratches.
4. Place the prism in the prism holder, with the highest-quality optical surface facing to your right. This is the side that the laser light will enter.

CAUTION Be very careful not to touch the laser mount when you install the prism. If it is knocked out of a position, it may require an engineer visit to fix.

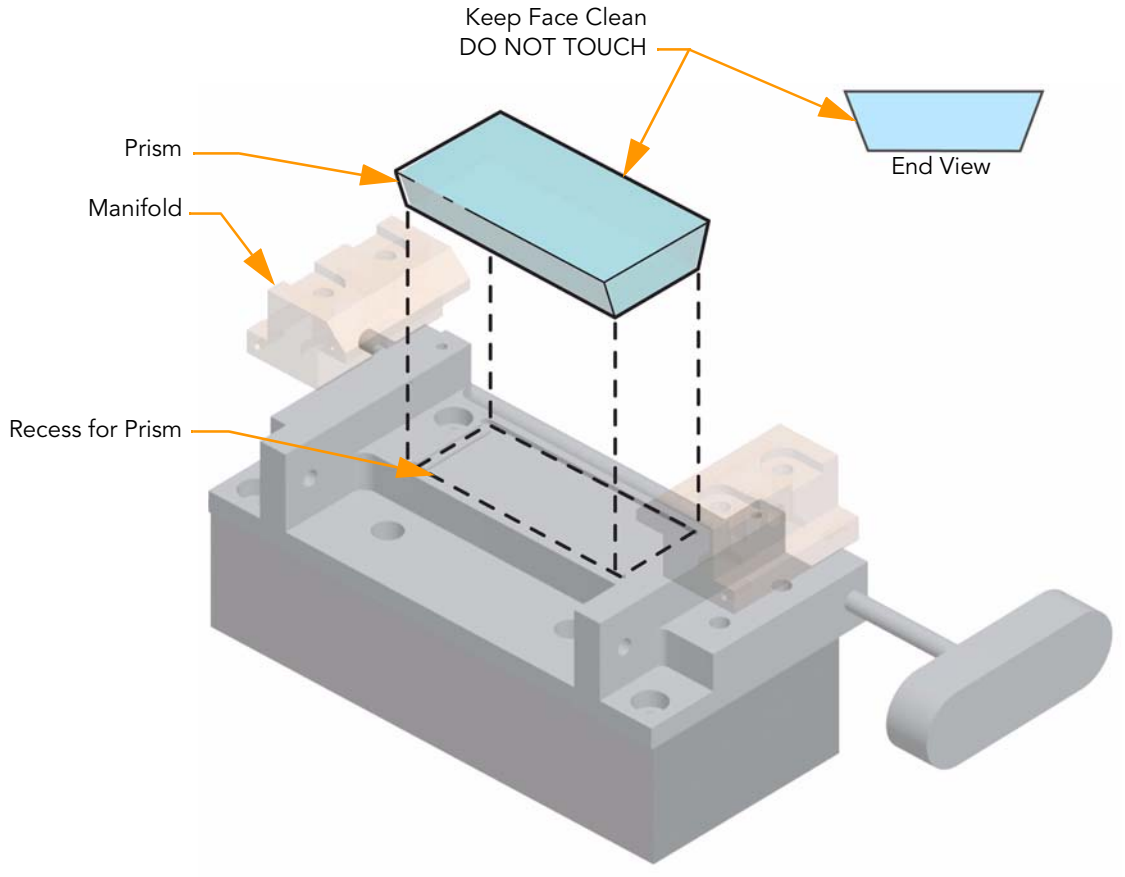

Figure 44 Loading the Prism

5. Test proper placement by gently attempting to slide the prism front to back and left to right. The prism should fit tightly and resist movement.
6. Proceed to *Cleaning and Installing the Flow Cell* on page 55.

Cleaning and Installing the Flow Cell

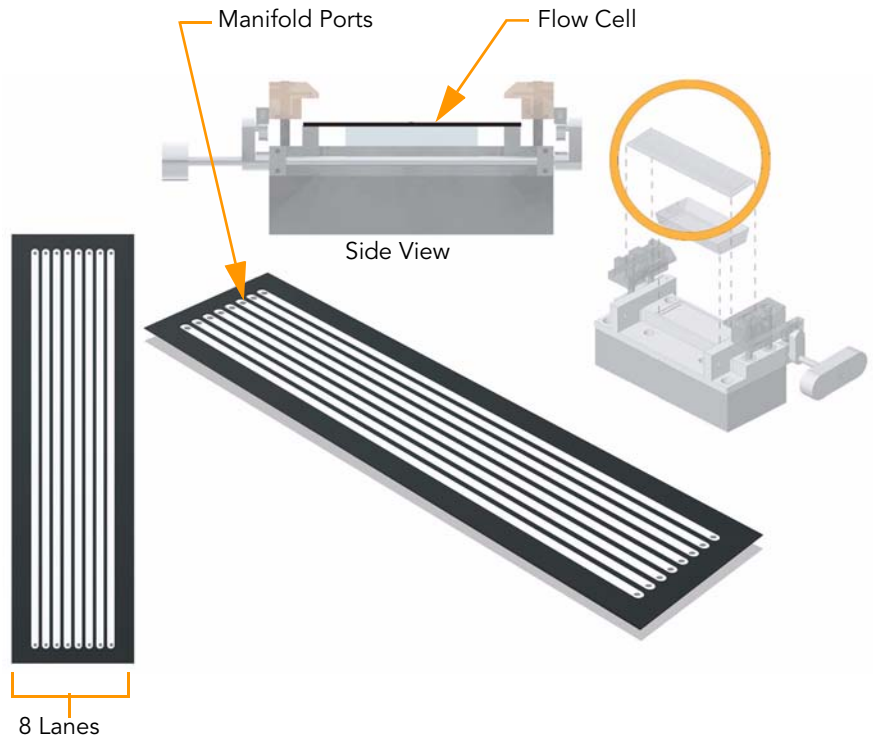

Figure 45 Flow Cell

Cleaning the Flow Cell

CAUTION

Work away from the inlets and outlets to avoid contaminating the inside of the lanes that contain the samples.

1. Put on new gloves.
2. If the flow cell appears very dirty after you remove it from the Cluster Station, wash it under deionized water before proceeding.
3. Place the flow cell on a lens cleaning tissue on the benchtop. Make sure that the inlet and outlet ports face *up*, to prevent liquid from flowing out of the lanes.
4. Fold a lens cleaning tissue to approximately the size of the flow cell. Wet the edge of the tissue with methanol or 100% ethanol.

5. Hold the edges of the flow cell with two gloved fingers.
6. Fold a fresh tissue, wet it, and wipe off each side with a single sweeping motion. Repeat, refolding the tissue with each wipe, until the flow cell is completely clean.
7. Use a new ethanol wipe to clean the bottom of the Peltier heater to make sure that no oil remains from a previous run.
8. Protect the flow cell from dust until you place it into the Genome Analyzer.

Loading the Flow Cell

The prism must be installed before you load the flow cell.

1. Place the flow cell on top of the front and rear mounting rails, with the inlet and outlet ports facing *up*. Press it gently against the rear stop.
2. Slide the flow cell along the rails to the right until you encounter the right stops.

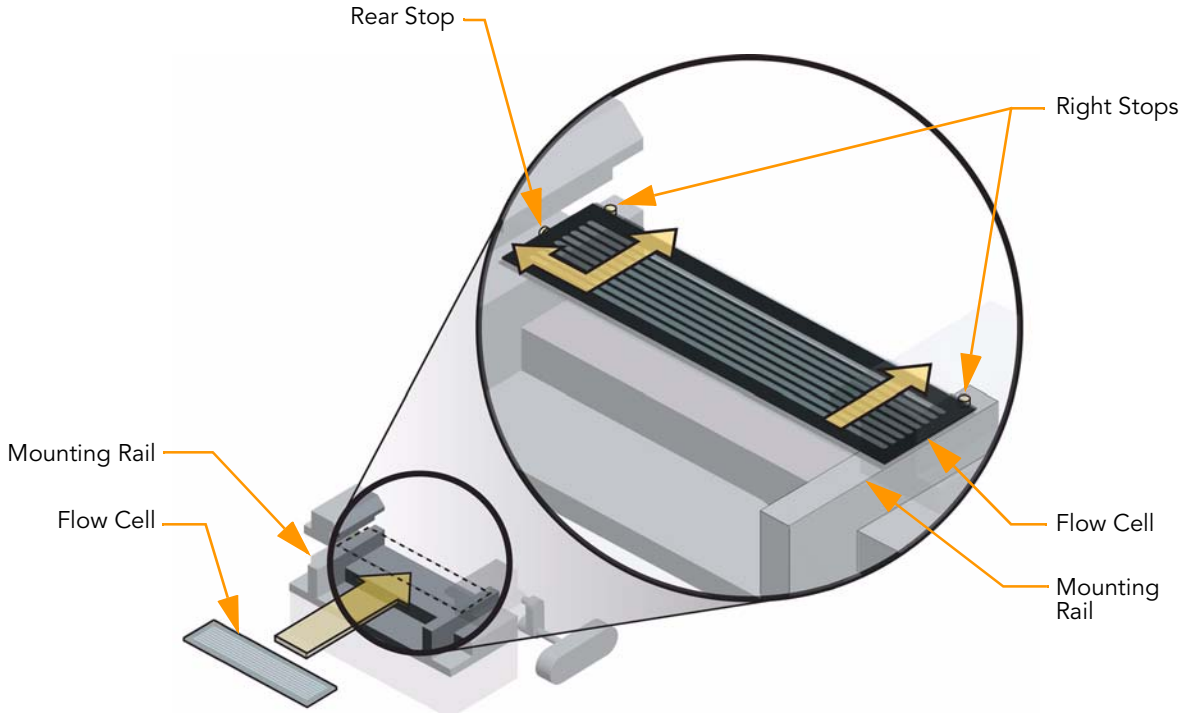

Figure 46 Loading the Flow Cell

3. Test proper placement by applying gentle pressure to the rear, then to the right to ensure the flow cell is pressed against both stops.
4. Using a lens cleaning tissue, gently apply pressure on the underside of the front manifold to absorb excess liquid.
5. Carefully rotate the manifold handle counterclockwise to lower the manifolds into place.

CAUTION

The manifolds are spring-loaded, and the cam that holds them up is steeply shaped. Be careful to control the spring action so that the flow cell is not damaged.

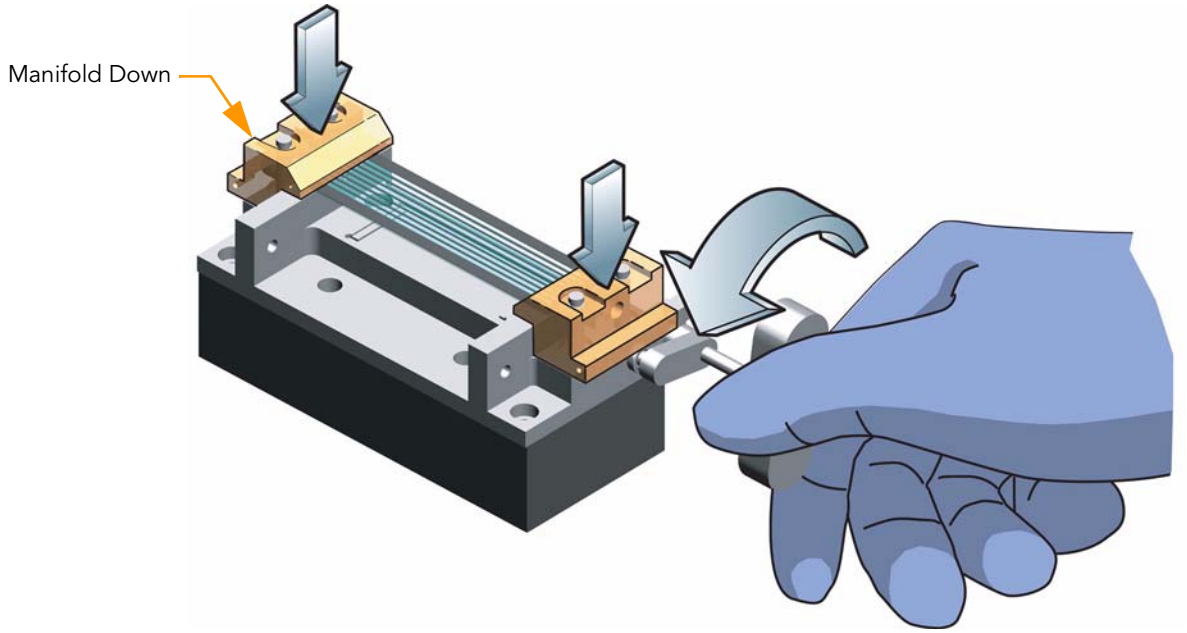

Figure 47 Lowering the Manifold

6. Press downward on both manifolds to ensure they have seated properly.

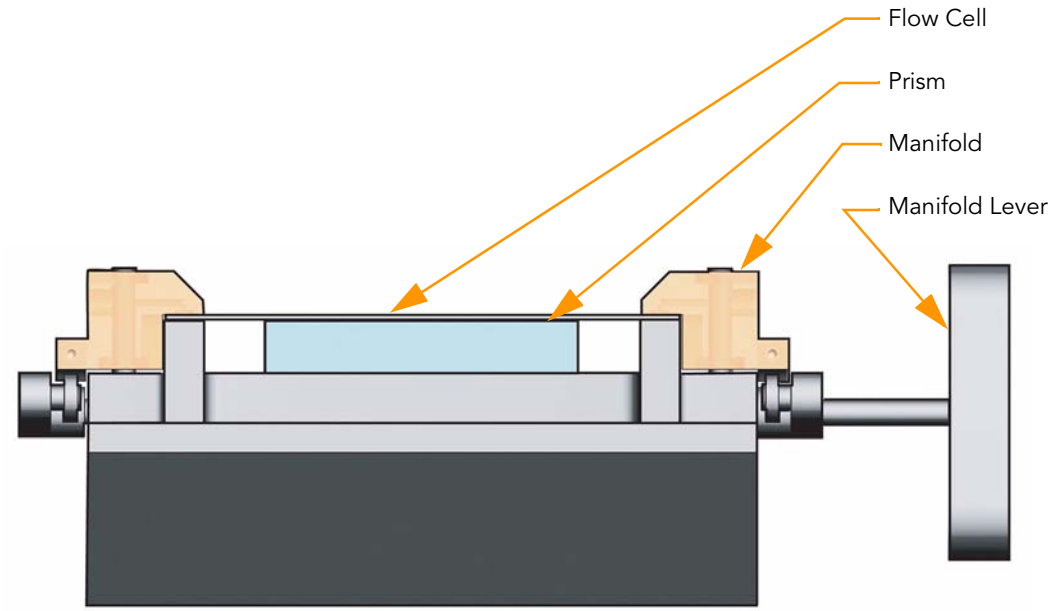

Figure 48 Flow Cell and Prism Loaded

7. Proceed to *Checking for Leaks and Proper Reagent Delivery* on page 58.

Checking for Leaks and Proper Reagent Delivery

Reagent delivery volumes during leak test are a key indicator of a stable fluid delivery system. The measured volumes must be within 10% of normal for optimal sequencing performance.

This procedure pumps wash solution through the flow cell to check for leaks. Excessive air bubbles through the lanes indicate leaks at the manifold.

1. Wipe the interface of the manifold and the flow cell with a lens tissue.
2. Bundle all of the lines together with parafilm, making sure to keep the ends even.
3. Place the bundle into a 1.5 ml tube.

   The next step is to pump 100 µl of the Incorporation Buffer (solution 5) through the flow cell.
4. Click the Manual Control/Setup tab.
5. In the Pump area, set the values as follows:

   Command: Pump to Flow Cell
   To: Flowcell
   Solution: 5
   Volume (µl): 100
   Aspiration Rate (µl/minute): 60
   Dispense Rate (µl/minute): 2000
6. With the cursor in the **Dispense Rate** box, press **Enter**.
7. Confirm that liquid is flowing properly through the flow cell by looking closely for any air bubbles being chased toward the rear manifold of each lane.

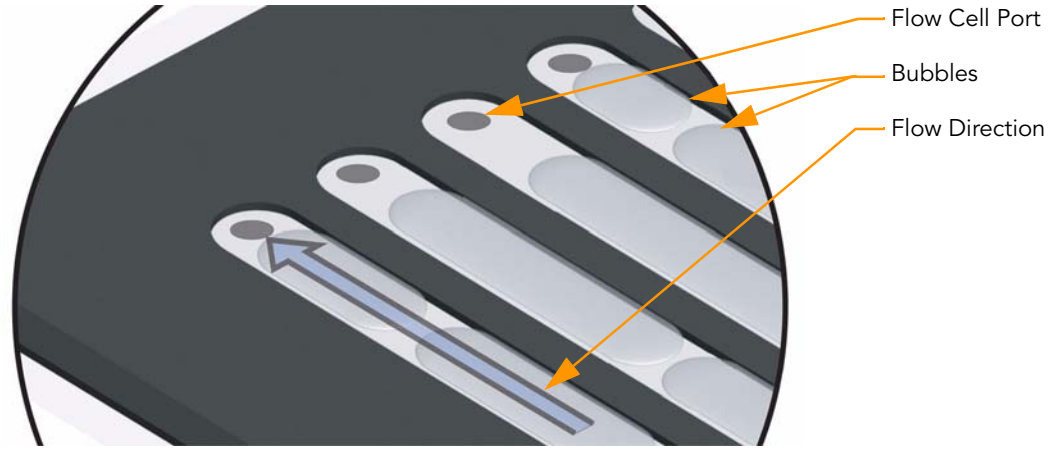

Figure 49 Checking for Bubbles

8. When the liquid has successfully displaced the air in all eight lanes, move on to check for liquid leaks. If bubbles persist, it might indicate that the flow cell is not properly seated on the flow cell stage.

   If air continues to enter the flow cell during the leak test, the most likely cause is a leak where the flow cell connects to the front manifold. Check the integrity of that connection and try the leak test again.

9. Lift the manifolds, clean the interface between manifold and flow cell with a moist lens tissue, and re-seat the flow cell.
10. Dry the flow cell and pump another 100 µl of the Incorporation Buffer (solution 5) through the system.
11. Measure the flow for each of the lanes three times. Record the measured volumes in the lab tracking worksheet.

    If the third measurement differs from the expected value by more than 10%, have the instrument checked by Illumina Technical Support.

    Once the system is leak free, the system is ready to run.
12. Proceed to *Applying Oil* on page 59.

Applying Oil

Immersion oil between prism and flow cell is a critical optical element. The layer of oil must be uniform and continuous to create total internal reflection through the layer of air between the prism and flow cell glass.

1. Click **Load Flow Cell** to move the stage forward.
2. Select **Instrument | Unlock Door**.
3. Carefully load 85 µl of immersion oil into a pipette tip, ensuring that there are no air bubbles in the oil in the pipette tip. Wipe the outside of the tip with a lens cleaning tissue.
4. Place the pipette tip on the prism at the gap between the top surface of the prism and the left side of the flow cell.

NOTE

Working from the left side of the flow cell helps to prevent oil from accumulating along the right surface of the prism where the laser light enters.

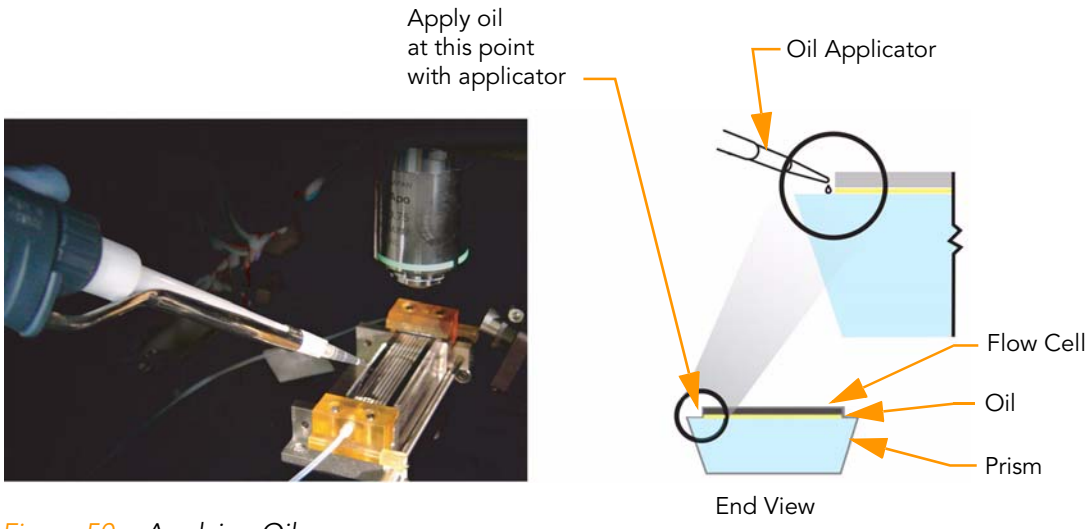

Figure 50 Applying Oil

5. Starting at the front-left corner, slowly dispense some of the oil and watch for it to spread under the flow cell and across the prism via capillary action.
6. Before the oil reaches the right side of the flow cell, slide the pipette tip slowly towards the rear, steadily dispensing more oil along the way.
7. Stop dispensing before the oil reaches the far right corner and wait a few seconds for the capillary action to complete.

   Add more oil only if the oil meniscus on the right side is not the full length of the flow cell. Be sparing with the oil; you should not have to use all of it.

Overloading the oil will cause a loss of illumination in lane 8, column 2, or could cause oil to wick over the imaging surface during the course of the run.

Applying too much pressure will move the prism out of the recessed space.

8. Ensure that the gap underneath the flow cell has a uniform layer of oil and that the right surface is clean. If anything is unsatisfactory, repeat the application.
9. Use an ethanol wipe to clean the bottom surface of the Peltier heater. This ensures that no immersion oil remains to contaminate the Peltier when it makes contact with the flow cell.
10. Close the instrument door.
11. Proceed to *Performing First-Base Incorporation* on page 60.

Performing First-Base Incorporation

In this step, you will incorporate the first nucleotide and then pause the system to set the focal plane.

1. Put the waste tubing of each lane into a 50 ml conical tube to determine the amount of fluid pumped through each lane during the sequencing run. Put the tubing through a small hole in the cap to minimize evaporation.
2. [Optional] Modify the FirstBase_v<#>.xml recipe file to change the number of tiles that will be imaged. For more information, see *Configuring Tile Selection* on page 110.
3. Open the Illumina Genome Analyzer Data Collection software and select **File | Open Recipe**.
4. Open the FirstBase_v<#>.xml recipe.
5. Click **Start**.

The software automatically makes a copy of the recipe file and stores it in the current run folder. If you need to stop work at any point, you can reopen the recipe from that location and continue from where you left off.

6. If prompted, browse to the sample sheet for this flow cell, and then click **OK**. (This feature may not be activated on all systems.)
7. Click **No** to dismiss the Autofocus Calibration dialog box. (You cannot perform autofocus calibration until the first fluorescently tagged base has been incorporated.)

   The run proceeds through the first phase of the recipe, which incorporates the first nucleotide.

   First-base incorporation chemistry takes approximately 35 minutes. At the end, a message indicates that the first-base incorporation chemistry is complete.

   The next step is to apply scan buffer and oil, and then determine the focal plane of the flow cell. This enables the software to automatically adjust its focus during the run.
8. Click **Cancel** as directed to pause the protocol. This allows you to control the software manually.
9. Proceed to *Loading the Flow Cell with Scan Buffer* on page 61.

Loading the Flow Cell with Scan Buffer

It is critical to introduce Scan Buffer to the flow cell before adjusting the focal plane.

1. Click the Manual Control/Setup tab.
2. In the Pump area, set the values as follows to pump Scan Buffer:

   Command: Pump
   To: Flowcell
   Solution: 3
   Volume (µl): 100
   Aspiration Rate (µl/minute): 60
   Dispense Rate (µl/minute): 2000
3. With the cursor in the **Dispense Rate** box, press **Enter**.
4. Proceed to *Adjusting Focus* on page 62.

Adjusting Focus

In this section, you will adjust the focal plane of the green laser along the X and Z axes.

During imaging, each tile is brought into focus by taking a focus image and finding the X-Y position of the laser spot in the image. The next Z position is calculated using the X and Y values and the predetermined focus calibration line.

Homing

When you initialize the Genome Analyzer, the lens orients itself using limit switches inside the instrument. Its default "home" XYZ coordinates are identified in the HCMConfig.xml file. These coordinates are specific to each machine and are configured during installation. Generally, the coordinates are set so that 0,0,0 is at the lower left corner and upper surface of the loaded flow cell.

The instructions in this section explain how to refine the focus so that the clusters are as sharp as possible. When you refocus and save the new origin points, the coordinates in the HCMConfig.xml file are updated and become the new defaults for that instrument.

WARNING

Never transfer an HCMConfig.xml file to any other Genome Analyzer. The coordinates will not be correct for that instrument, and the lens, flow cell, or other equipment may be damaged or broken when the lens homes itself.

Manual Controls

In the Manual Focus step, you take photographs from different positions and adjust focus along the X, Y, and Z axes as necessary.

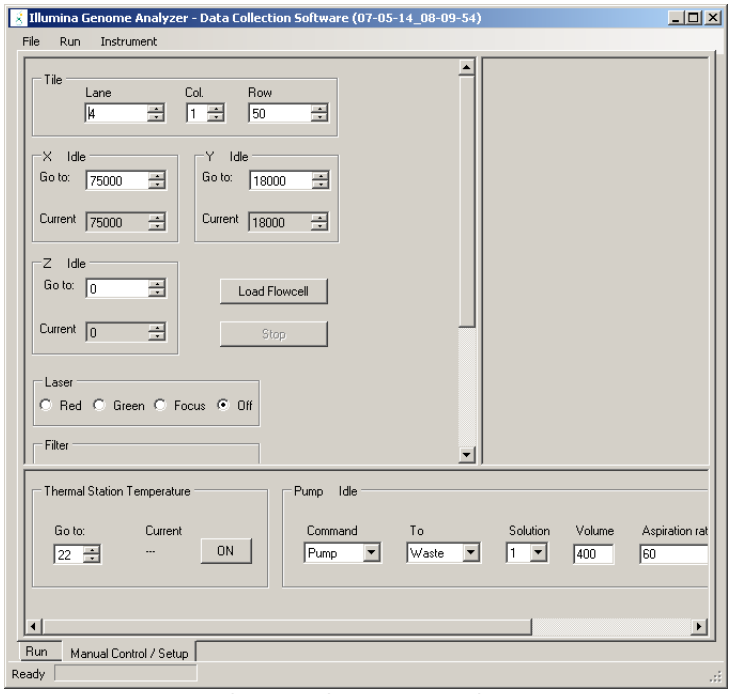

Figure 51 Manual Control/Setup Window

The following table describes the areas of the Manual Control/Setup window that you use for manual focus.

Table 9 Manual Focus Controls

Area of the Screen	Description
Tile	Enter lane, column, and row coordinates to move the objective lens to a certain tile. The XY zero point for flow cells is typically Lane 4, Column 1, Row 50. This is at the left edge of the flow cell on the X axis, and halfway back on the Y axis.
X/Y (µm)	Shows the current position of the flow cell underneath the objective lens. Because of minute variations between flow cells, you need to fine-tune the X value so that the edge of lane 1 is at the center of the image after you load a new flow cell. You can adjust the position by entering new values into the **Go To** boxes and pressing **Enter**. The laser remains stationary, and the flow cell moves underneath it. All directions below are given as if you were standing in front of the instrument compartment. Increase X to move the laser toward the right of the flow cell (the stage moves toward the left). Increase Y to move the laser toward the back of the flow cell, near the output ports (the stage moves toward the front). Note: You do not need to adjust this unless there is a significant hardware change, in which case an engineer will reset it. After you set the X value correctly, you will re-zero the X coordinate using the **Instrument | Set Coordinate System** menu.
Z (nm)	Shows the current position of the objective lens relative to the flow cell. When you insert a new flow cell, you need to refocus the lens by changing the Z position so that the cluster images are sharp. You can adjust the position by entering new values into the **Go To** boxes and pressing **Enter**. The flow cell remains stationary, and the lens moves up and down. Increase Z to move the lens away from the flow cell vertically. Decrease Z to move the lens closer to the flow cell. Ideally, the value of Z at the focal position should be zero (0). The farthest you can safely move below the focal point is 40,000 nm (Z = -40000). The position of the flow cell surface varies from one flow cell to another. Sometimes you have to move down 20,000 nm to find the focal plane of a new flow cell. When you find the focus, re-zero the Z axis for that run. Note: The focal position on the Z axis must be greater than -20,000 (as noted above, it should be exactly zero), or the laser will not have sufficient room to move down while tracking focus position during the run.
Laser	Controls which laser is used during the exposure. **Red laser**—For use with A and C filters. **Green laser**—For use with G and T filters. **Focus**—Used by Illumina Technical Support and during autofocus calibration to illuminate the focus spot. **Off** (default)—Turns off the laser for the photo, using only ambient reflected light for the photo. Support scientists sometimes use this to check the optical path.
Filter	Moves the filter wheel to view only the light from a particular base (A, C, G, T). If you select a filter base, the Laser must be set to the corresponding color (e.g., red for A). If you selected the Focus laser, set **Filter** to None.
Camera	**Exposure**—Lets you set the exposure time in milliseconds. **Take Picture**—Click to take a picture using the current configuration. **Start Video**—Click to display a series of images in a sequential loop (like a movie), using the current configuration. Click the button again to stop the video.

Adjusting the X-Axis

1. The first step is to check the position of the flow cell along the X axis. The zero point should be at the left edge of the flow cell.
2. Click the Manual Control/Setup tab.
3. In the Tile area, set the coordinates to Lane 4, Column 1, Row 50. This is the approximate center of the flow cell.
4. Set the **Go To** values in the X, Y, and Z areas as follows:

   Z (nm): 0 (zero). This assumes that you reset the Z position to zero when you focused it during the last run. For more information, see *Adjusting the Z-Axis* on page 67.

   X (µm): 0 (zero).

   Y (µm): Same as the value displayed in the **Current** box.

   This moves the stage to a position near the edge of the flow cell. The left edge of the flow cell is underneath the objective.
5. Set the following values:

   Laser: Green

   Filter: None

   Exposure (msec): 40
6. Click **Take Picture**.

   The edge of the flow cell appears on the screen. The crosshair represents the center of the image at x=500, y=500 pixels. One pixel is approximately 340 nm.

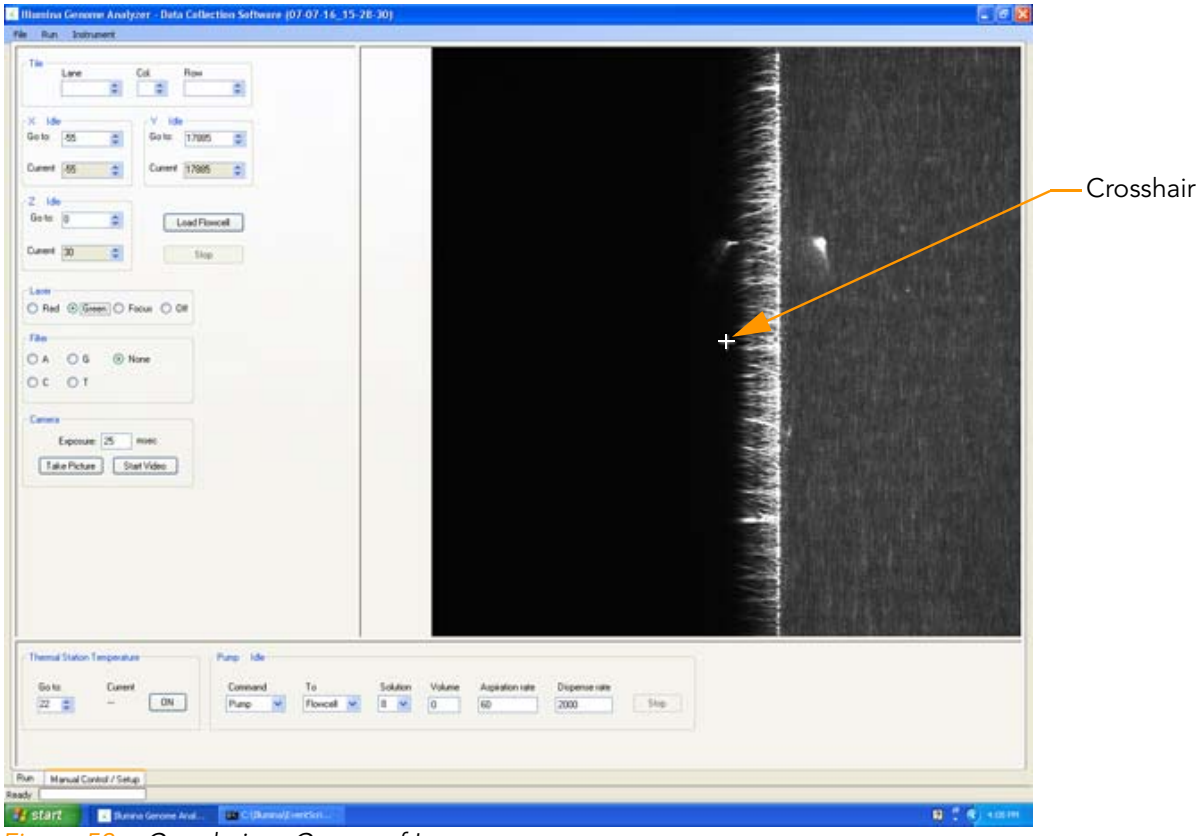

Figure 52 Crosshair at Center of Image

The left edge of the flow cell should be close to the center of the image. To identify the distance between the edge of the flowcell and the crosshair, use your mouse to position the arrow over the edge of the flow cell. The pop-up window will reveal the position of the arrow in pixels.

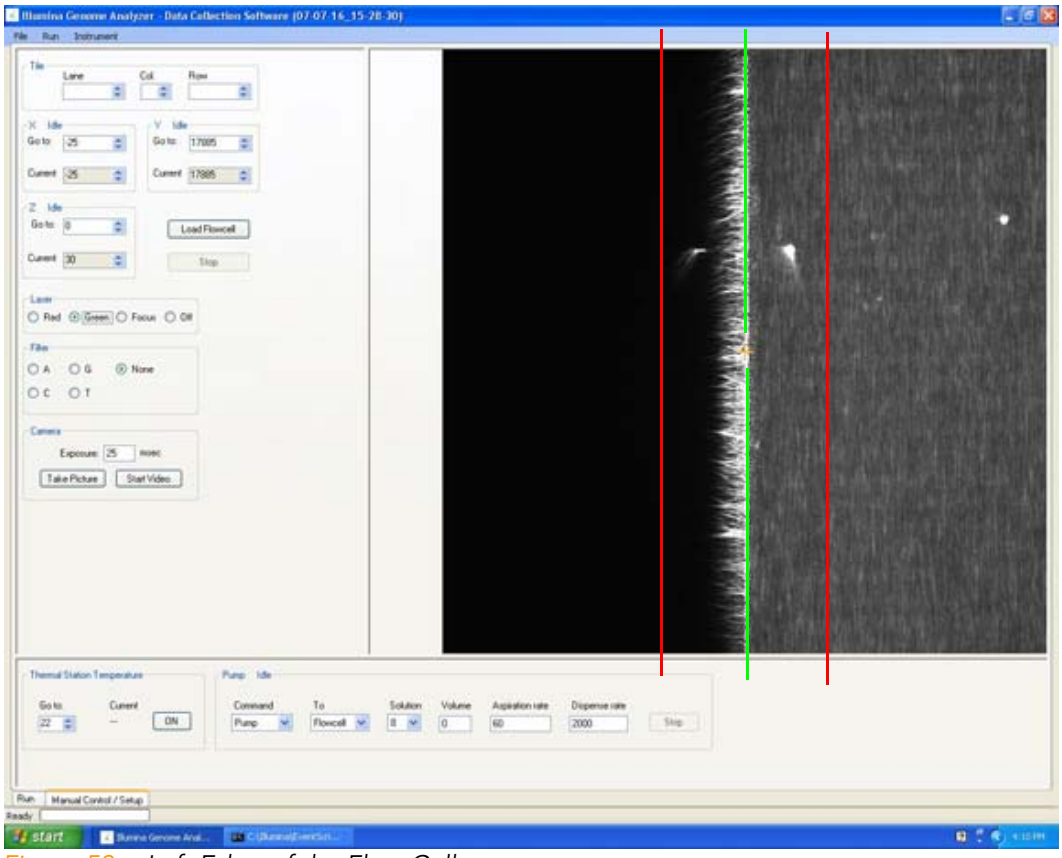

Figure 53 Left Edge of the Flow Cell

7. If the edge is more than 100 pixels (for a two-column setup) or 3–5 pixels (for a three-column setup) from the vertical crosshair, move the stage in the X axis to bring the edge closer to the crosshair. (Increase the X value to move the edge of the flow cell to the left; decrease it to move the flow cell to the right.)

NOTE

You cannot change the X origin by more than 2000 µm at a time.

8. Select **Instrument | Set Coordinate System | Set Current X as Origin**.
9. Click **OK** to confirm that you want to reset the coordinates.
   This automatically adjusts the values in the HCMConfig.xml file.

Adjusting the Y-Axis

Do not adjust this.

Only Illumina Field Service should ever adjust the Y axis. It is not necessary unless you replace a manifold, the XY stage, or the Z stage and optical column.

Adjusting the Z-Axis

In this section, you take photos over Lane 4, 1, and 8 (in that order) and check the focus to determine the optimal position of the flow cell along the Z axis. You should adjust the X axis before performing this procedure.

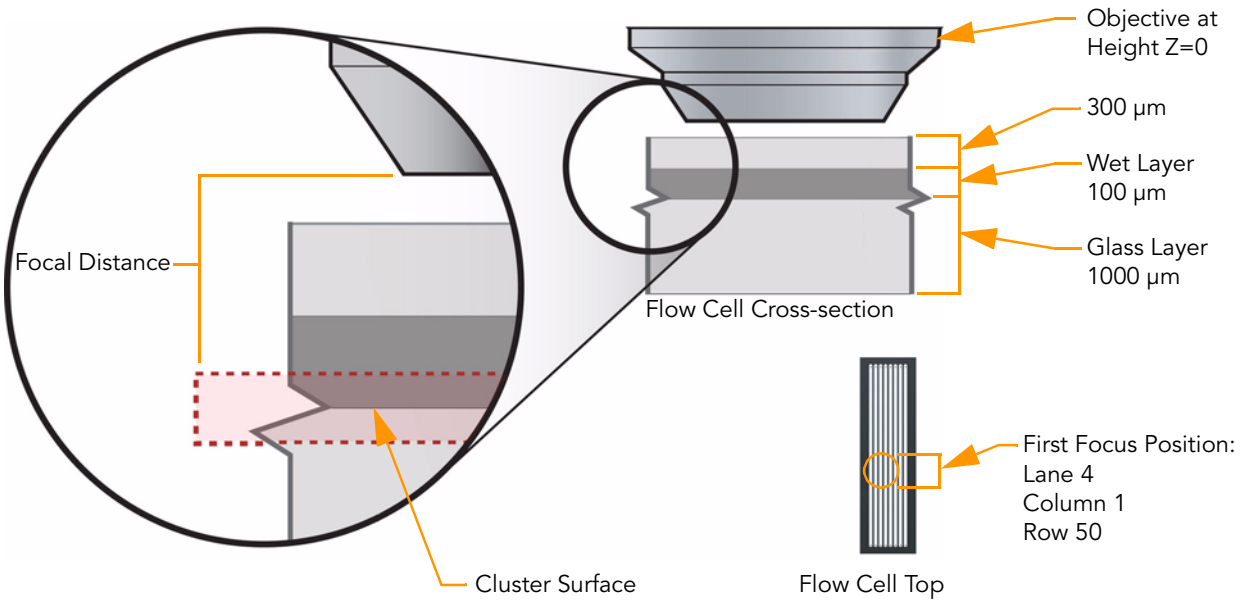

Figure 54 Focusing Z-Axis

CAUTION Minimize the number and duration of exposures during manual focus on a given tile. Photo bleaching will start to diminish the intensity of the cluster signals after a few seconds of total exposure.

1. In the Tile area, set the coordinates to Lane 4, Column 1, Row 50. This is the approximate center of the flow cell.

NOTE Typically, the center tile is used for setting the focus but you may prefer to move closer to the start of imaging at Row 5. Ensure that the calibration curve is of good quality and the flow cell tilt is less than 15,000 nm.

2. Set the following parameters to sharpen the cluster images:
   Laser: Green
   Filter: T
   Camera Exposure (msec): 400
3. Click **Take Picture**.
4. Use the reference images shown here to decide what direction to move the laser. Start with a move no larger than 5000 nanometers. Subsequent moves can be smaller as you approach the optimal focus position.
5. Each time you move the Z position, take a new photo and check it.

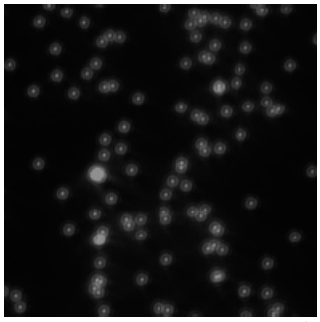

Figure 55 Lens Too High

A distinct halo effect, especially around the smaller (less intense) clusters, is characteristic of images acquired with the lens just above optimal focus position. Lower the Z value.

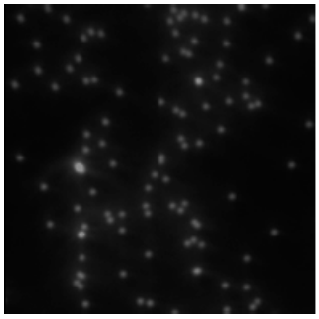

Figure 56 Lens Too Low

A uniform loss of sharpness and intensity is characteristic of images acquired with the lens just below the optimal focus position. Increase the Z value.

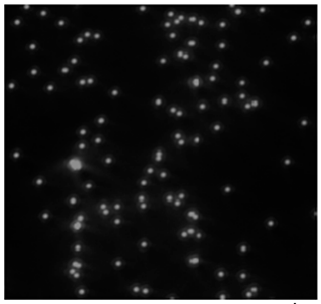

Figure 57 Lens Properly Positioned

6. Adjust the Z coordinate as described above, until the clusters are in sharp focus.
7. Evaluate the image to determine whether first-base incorporation was successful.
8. Do one of the following:
   - If first-base incorporation failed, discontinue the run and perform the post-run instrument wash (*Post-Run Wash* on page 71).
   - If first-base incorporation was successful, record the Z position for Lane 4 in the lab tracking worksheet.

9. Select **Instrument | Set Coordinate System | Set Current Z as Origin**.
10. Proceed to *Checking Quality Metrics* on page 69.

Checking Quality Metrics

Performing Autofocus Calibration

In autofocus calibration, the system takes a series of photos and performs an analysis that will enable it to focus correctly on each tile during the run. After starting the calibration, all you need to do is watch for warnings in the Result window.

1. Click the Run tab.
2. Highlight the UserWait step right before the "Incorporation" line in Cycle 1, if it is not already selected.
3. Click **Resume**.
4. Click **Yes** at the prompt.

   The software automatically performs an autofocus calibration based on the Z value that you determined during manual focus.
5. After calibration, the following window appears, showing the calibration values.

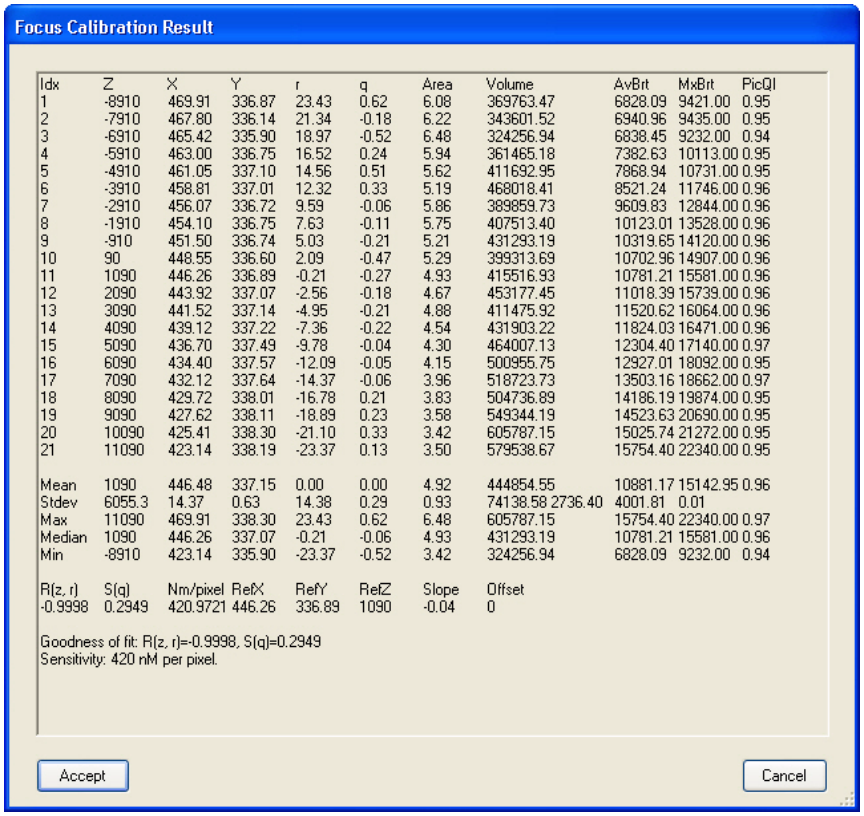

Figure 58 Autofocusing

6. Check the following specifications:

   Goodness of fit: ± 0.99XX

   Absolute value of the sensitivity: 400–450.

   A warning appears at the bottom of the window if either parameter is out of range. You might see any of the following warnings:

   - Warning: CurveFit received _____ images, expecting 21
   - Warning: Sigma(q) = _____ exceeds allowed threshold 0.5
   - Warning: Correlation coefficient R(z, r) = _____ is less than allowed threshold 0.95
   - Warning: Focus Calibration sensitivity is too low. Nm/pixel _____ exceeds allowed threshold 500.00
   - Warning: Mean spot picture quality = _____ is less than allowed threshold 0.90. Increase exposure.

7. Do one of the following:
   - If both values are within the specified range, click **Accept**, and then click **OK** at the prompt.
   - If either or both of the values does not meet the specification, move to another tile, refocus, and try again.

Surface contamination is the most common cause for poor autofocus calibration. For example, a little oil may have gotten on the surface when you applied oil to the flow cell/prism interface.

   - If the calibration fails again, remove the flow cell and prism. Clean and reload the flow cell and prism, and check for leaks. Do not repeat the first-base chemistry step. Instead, proceed directly to loading scan buffer (*Loading the Flow Cell with Scan Buffer* on page 61) and continue from there.
   - If the calibration fails again, consult Illumina Technical Support.

8. Observe the images in the next cycle as well to determine if they stay in focus. If the focus is poor, stop the run and refocus before all of the images are collected.

   For additional information about autofocus calibration that will help you determine whether to continue, run a Laser Spot Metric Report in Run Browser. For instructions, see *Laser Spot Metrics: Measuring Autofocus Performance* on page 83.

9. Proceed to *Viewing Data in Run Browser* on page 70.

Viewing Data in Run Browser

Run Browser is a report tool that launches automatically after first-base incorporation. You should always load the run log file(s) to assess the quality of the data and decide whether to complete the run.

To view and analyze the data, and learn about Run Browser, follow the instructions in Chapter 5, *Run Browser Reports*.

For more information about Goldcrest, the utility that collects data for Run Browser, see Chapter 4, *Using Goldcrest*.

After analyzing first-base incorporation data, proceed to *Completing the Run* on page 71

Completing the Run

If you are satisfied with the results of the first-base incorporation, follow these instructions to complete the run. A full sequencing run may take 48–72 hours.

1. Select **File | Open Recipe**.
2. Open the 26Cycle_v<#>.xml recipe.

   You may also run the 18Cycle_v<#>.xml or 36Cycle_v<#>.xml recipes.
3. Click **Start**.
4. When prompted, click **OK** to accept the name of the run folder. For more information about run folders, see Appendix A, *Run Folders*.
5. When the Autofocus Calibration dialog box appears, click **No** (you've already calibrated). The Genome Analyzer resumes sequencing.
6. Top off the Scanning Mix before cycle 26. If you are running more than 26 cycles, top off the Scanning Mix when 26 cycles are remaining in the run. The tube should be approximately one-third empty after running for six or seven hours.

You may top off the Scanning Mix when the instrument is imaging. Do not top off the Scanning Mix when the instrument is about to pump.

7. When the run is complete, proceed to *Performing Post-Run Procedures* on page 71.

Performing Post-Run Procedures

When the run is complete, notify the appropriate personnel that data are available for analysis.

Weigh all of the reagent bottles and record the results in the lab tracking worksheet.

Weigh all of the fluids that have been pumped through the eight lanes and record the results in the lab tracking worksheet.

Post-Run Wash

After every run, you must perform a thorough instrument wash. The wash flushes 4 ml of instrument wash reagents through each reagent port. Run time is approximately 45 minutes. Perform post-run washes immediately after a run so that they do not interfere with the next run setup.

1. Load the instrument with solutions as follows:
   50 ml Instrument Wash solution for port positions 1, 6, 3, 4, 5, and 7
   250 ml deionized water for port position 2
   Rotate the tubes while holding the caps stationary, to prevent crimps and twisting in the liquid delivery lines.
2. Bundle all waste tubes with parafilm, making sure to keep the ends even.
3. Place the bundled tube ends into a pre-weighed 50 ml conical tube.
4. Click the Run tab.
5. Select **File | Open Recipe**.
6. Open the PostWash_v<#>.xml recipe file.
7. Click **Start** and enter a file name.
   The wash cycle runs for approximately 45 minutes.
8. After the wash, weigh the tube and record the results in the lab tracking worksheet.

Using wash reagents other than the Instrument Wash Solution in the Sequencing Kit, or failing to perform the wash cycle at the recommended intervals, may void the warranty.

Chapter 4

Using Goldcrest

Topics

Introduction

Goldcrest is a real-time utility for collecting image metrics during runs. Following first-base chemistry and autofocus calibration, the Genome Analyzer automatically images 48 images (six tiles per lane). The Goldcrest software extracts this data and stores it in <install directory>\Service\<DateTimeStamp>. Goldcrest performs statistical operations on the data and produces cluster intensity files, average statistics, and image quality values which act as input material for the Run Browser report generator.

Delete all files in the <install directory>\Service directory every 5 or 10 runs.

Goldcrest is already installed on your system when you receive it. It runs during first-base incorporation, to provide the information that will help you determine whether to complete the run.

The only thing you have to do to set up Goldcrest is to update the location of the default_offsets file.

Goldcrest has the same function and image-processing algorithms as the Firecrest module in the Analysis Pipeline package. Goldcrest v0.6 is equivalent to the Firecrest module included in the 0.2.1 version of the pipeline.

Updating the Offsets File

The offsets file (default_offsets.txt) corrects for any slight misalignments between the four image channels (A, C, G, T) that could lead to incorrect cluster counts. A default offsets file is provided during installation, but the optical path of the instrument may have shifted during shipping, thus requiring an updated default offsets file.

When you run the Analysis Pipeline after the completed run, it automatically generates a default_offsets.txt file that is calibrated to your particular instrument. To ensure top quality results, you must copy this file to a location that Goldcrest can access.

Update this file on a regular basis. In addition, update it after any optical repair or upgrade performed on the instrument.

To Update the Offsets File

1. Navigate to <install directory>\Goldcrest_v<#>\Release and open the ConfigPipe.xml file in a text editor.
2. Do one of the following:

- (Recommended) If you have network access to the server location where run data is copied and stored for later analysis, change the following line to point to the location of the default_offsets.txt file created by the Analysis Pipeline. By default, this is as follows:
  `<offsets_file>\\<server name>\Runs\Instruments\<computer name>\default_offsets.txt</offsets_file>`
  where <server name> is the location of the data storage server and <computer name> is the name of the instrument computer.
- If you do not have network access, obtain the default_offsets file generated by the Analysis Pipeline and copy it to C:\Instrument\. Change the <offsets_file> line as follows:
  `<offsets_file>C:\Instrument\default_offsets.txt</offsets_file>`

Goldcrest Output

Goldcrest runs in a command window in the background during first-base incorporation. It monitors the contents of the data collection run folder and analyzes image files in sets of four (A, C, T, G) as they appear. The statistical information it generates for each tile is saved to the *bro.xml file that Run Browser uses for input.

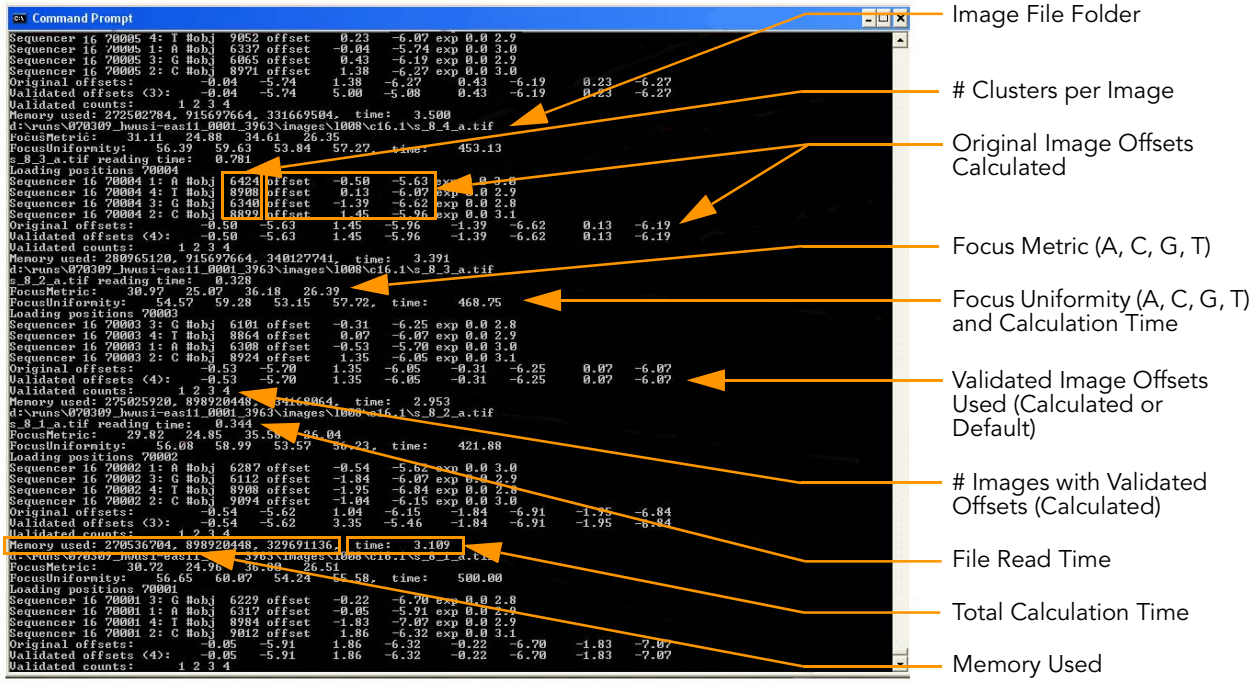

Figure 59 Goldcrest Command Window

Every cluster is represented by a line in the file that identifies raw intensity values for the A, C, G, and T image channels. The files contain from 5,000 to 40,000 lines of data depending on the cluster density.

In raw signal, the T dye is the brightest, followed by G, C, and A. The values in this file are neither matrix corrected nor adjusted for phasing.

Result Files

Goldcrest result files are saved in <Run Folder>\Data\Goldcrest\Firecrest. They are similar to Firecrest output. The files include:

- **s_#_####_idx.txt**—Lane number; Tile number; X, Y integer positions.
- **s_#_####_##_int.txt**—Cluster intensities for A, C, G, T channels. These are the raw signal, and are not matrix corrected or normalized.
- **s_#_####_##_nse.txt**—Cluster background noise for A, C, G, T channels.
- **s_#_####_pos.txt**—X and Y coordinates of each cluster.
- **s_#_####_##_qcm.xml**—Confidence level for base calls
- **s_#_####_##_#_clu.txt**—Number and size of clusters

# denotes the lane number. #### denotes the one-digit column number plus the three-digit tile number.

Files for Run Browser

The following files are used by Run Browser to generate reports:

- **s_#_##_bro.xml**—One file per lane (#), per cycle (##)
- **RunLog_MM-DD-YY_HH-MM-SS.xml**—One or more log files from the run
- **Focus.2.1.csv**—Comma-delimited file containing focus metrics

Chapter 5

Run Browser Reports

Topics

Introduction

Run Browser is a utility that lets you assess the quality of run data on the dedicated Genome Analyzer workstation, without the need to copy the data to a server location or perform a complete pipeline analysis. You can view the data quickly in a summary window, or generate reports that you can print, save, or export.

Run Browser's primary function is to provide access to the data from first-base incorporation, so that you can decide whether or not to complete the run. By default, Run Browser launches automatically after the completion of first-base incorporation.

Run Browser uses the following Goldcrest-generated files:

- s_#_##_bro.xml
- RunLog_MM-DD-YY_HH-MM-SS.xml
- Focus.2.1.csv

For more information about Goldcrest, see Chapter 4, *Using Goldcrest*.

User Interface

The Run Browser has two windows: the Flow Cell window and the Report window. The Flow Cell window provides a graphical interface for quickly gathering data and seeing it in color with interactive tooltips, while the Report window enables you to create textual reports on the same data.

Flow Cell Window

When you open the Run Browser, the Flow Cell window appears. Navigate to the run folder and open all of the log files from the run to display the aggregate data.

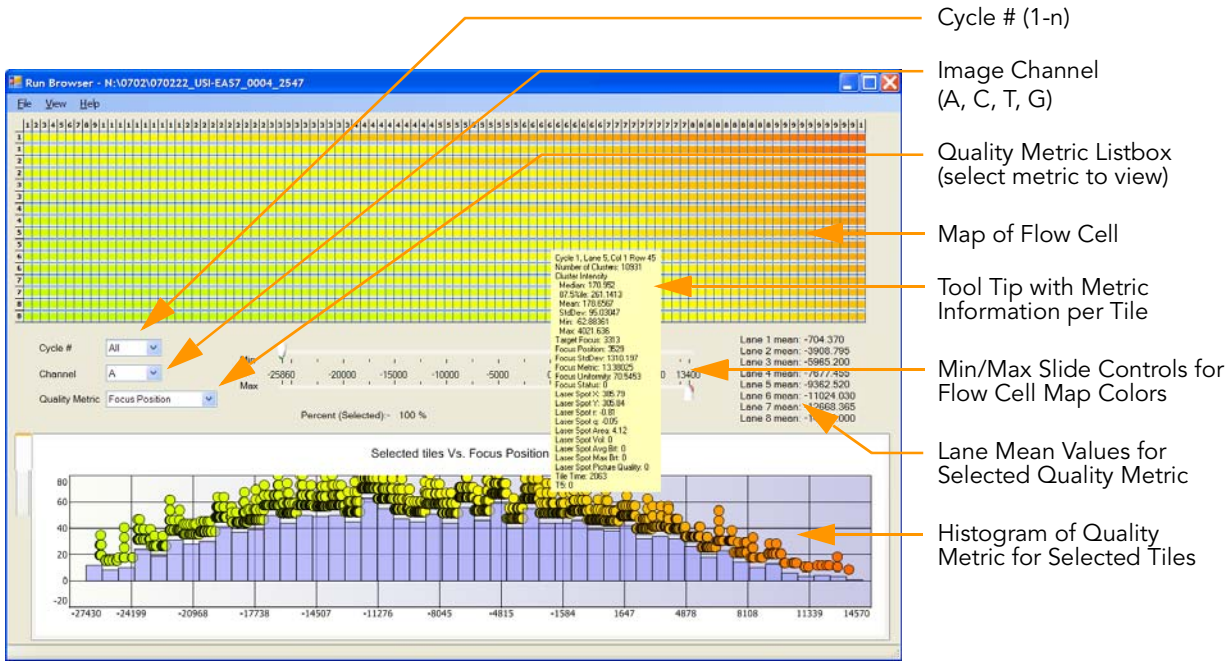

Figure 60 Flow Cell Window

Report Window

After opening the log file, select **View | Report** to open the Report window. From here, you can generate text reports that you can then print, save, or export.

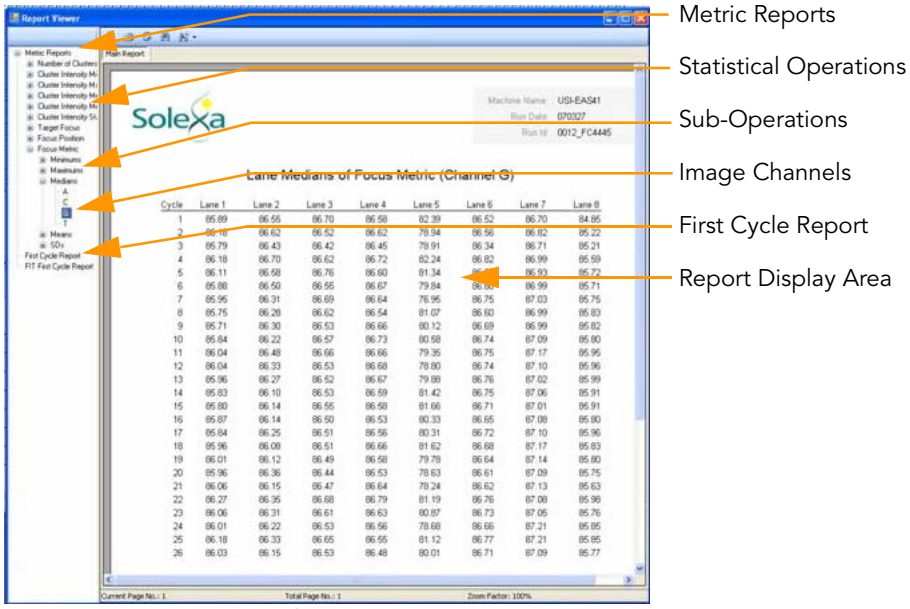

Figure 61 Report Window

Report Types

Run Browser provides two types of reports: a First-Cycle report and a set of metric reports.

First-Cycle Report

The First-Cycle report contains summary data about first-base incorporation. Illumina recommends generating a First-Cycle report after performing first-base incorporation and using it to make an informed decision about whether to continue the run.

The report lists metrics for the cluster number counts, intensity values, focus metric, focus position, and flow cell tilt.

NOTE

The focus metrics may not be accurate for high-density tiles (> 15K).

Metric Reports

Run Browser metric reports describe the results of statistical operations performed on the tiles in a lane during a cycle. The possible statistical operations are:

- Minimums
- Maximums
- Medians

- Means
- Standard Deviations (SDs)

For recommendations on how to use these reports to assess run data, see *Using Reports to Assess Run Data* on page 81.

Using Reports to Assess Run Data

Cluster Metrics: Measuring Cluster Quality

The following reports help you evaluate cluster quality before continuing a run. Each one performs a statistical operation on an individual image channel (A, T, C, G) during a given cycle.

Table 10 Measuring Cluster Quality

Statistical Operation	Description
Cluster Intensity Median	The median intensity of the selected channel, per tile. This metric is useful when all clusters begin with the same base and all the signal resides in one channel. It dates from when all sequencing began with a T base.
Cluster Intensity Mean	The mean intensity of the selected channel, per tile. This metric is useful when all clusters begin with the same base and all the signal resides in one channel. It dates from when all sequencing began with a T base.
Cluster Intensity Minimum	The minimum cluster intensity of the selected channel, per tile. This metric is useful when all clusters begin with the same base and all the signal resides in one channel. It dates from when all sequencing began with a T base. This metric has little value when the first base is diverse. It will report negative values because of background subtraction.
Cluster Intensity Maximum	The maximum cluster intensity of the selected channel, per tile.
Cluster Intensity Standard Deviation	The standard deviation of the selected channel, per tile. This metric is useful when all clusters begin with the same base and all the signal resides in one channel. It dates from when all sequencing began with a T base. This metric has little value when the first base is diverse and equally distributed.
Cluster Intensity 87.5%	The 87.5th percentile of the cluster intensity for the selected channel, per tile. This metric assumes approximately equal distribution of the beginning base for each cluster. It represents the median value of the "true" dye signal. See Table 11 for benchmarks that help you decide whether to continue the run.

Confidence Levels

Table 11 Confidence Levels for 87.5%ile Intensities

Confidence Level	Intensity			
	A	C	G	T
High Confidence	> 8,000	> 10,000	> 10,000	> 12,000
Reasonable Confidence	> 5,000	> 5,000	> 5,000	> 6,000
Low Confidence	< 3,000	< 3,000	< 3,000	< 5,000

Focus Metrics: Measuring Image Quality

The following reports help you evaluate the image quality of the run. The data come from the *bro.xml file, where Goldcrest records information on the Z-stage position and image quality of each tile.

Table 12 Measuring Image Quality

Statistical Operation	Description
Target Focus	The Z-stage position of the target focus, as calculated by the auto-focus algorithm. The position is the same for all channels.
Focus Position	The actual Z-stage position that the hardware reports to the control software after moving. Compare this metric with the Target Focus values to evaluate the autofocus control loop. The position is the same for all channels. The focus position should stay relatively constant between cycles. If there are significant jumps, it may indicate that the lens is going in and out of focus.
Focus Standard Deviation	The standard deviation of the Z-stage position of a tile over all the cycles in the run log(s) currently loaded in Run Browser. This is the only focus metric that is calculated per tile over all cycles. If you see a large variation in the Z-stage positions (StdDev > 6,000 nm), it indicates poor focus control or the introduction of a significant number of bubbles into the system.
Focus Metric	A number that represents the sharpness or focus quality of the image. It is calculated separately for each channel. A high-quality image will have a focus metric above 65. The focus metric is not accurate for tiles with cluster density greater than 15,000.
Focus Uniformity[a]	An assessment of the focus quality across the tile image, calculated separately for each channel. The software divides the tile into a 3x3 grid, calculates the focus metric for each square, and divides the minimum value by the maximum value. Tiles with even focus have focus uniformity numbers of about 90–95.
Focus Status	A number corresponding to a warning message (see Table 13), if there is one, for each tile. You should take these numbers into account when deciding whether or not to complete the run.

a. The T5 metric is an old focus uniformity metric that is no longer in use.

Warning Messages

Table 13 Focus Status Warning Messages

Number	Warning Message	Move Z Axis?
0	No warning message.	Yes
1	Poor laser spot quality or parameter. Spot has high q residual.	Yes
2	Poor laser spot quality or parameter. Spot has outlier spot chars.	No
3	Poor laser spot quality or parameter. Spot has high q residual and outlier spot chars.	No
4	Poor laser spot quality or parameter. Spot has low picture quality.	No
5	Poor laser spot quality or parameter. Spot has high q residual and low picture quality.	No
6	Poor laser spot quality or parameter. Spot has outlier spot chars and low picture quality.	No
7	Poor laser spot quality or parameter. Spot has high q residual, outlier spot chars, and low picture quality.	No

Laser Spot Metrics: Measuring Autofocus Performance

These reports help you evaluate the success of the autofocus calibration (see *Performing Autofocus Calibration* on page 69), and also indicate when there is air in the system.

Table 14 Measuring Autofocus Performance

Statistical Operation	Description
Laser Spot X	The X pixel position of the center of light of the autofocus (AF) laser spot on the tile image for the selected cycle.
Laser Spot Y	The Y pixel position of the center of light of the AF laser spot on the tile image for the selected cycle.
Laser Spot r	The r value calculated from the image of the selected cycle. Large changes in r can indicate air in the system.
Laser Spot q	The q value calculated from the image of the selected cycle. Large noise in Q can represent poor setup of the autofocus system.
Laser Spot W	This metric is not currently in use.
Laser Spot H	This metric is not currently in use.

Table 14 Measuring Autofocus Performance

Statistical Operation	Description
Laser Spot A	The area, in pixels, of the detected laser spot above the detection threshold.
Laser Spot P	This metric is not currently in use.
Laser Spot D	This metric is not currently in use.
Laser Spot Vol	The average brightness (above the detection threshold) multiplied by the area of the primary laser spot. A large increase in volume indicates air in the system.
Laser Spot Average Brightness (Avg Brt)	The sum of the gray values of the pixels in the laser spot, divided by its area.
Laser Spot Maximum Brightness (Max Brt)	The maximum gray value of the pixels in the laser spot, divided by its area.
Laser Spot Pic Quality	The average of the normalized autocorrelation of the image with itself, with shifts of unit pixel to the left and down. If the image is noisy, the measure will be low because the noise does not correlate with itself.

Other Metrics

Number of Clusters

The Number of Clusters metric shows the total number of identified clusters in each tile. It is the only cluster metric that is not specific to an image channel. If the offset file is correctly calibrated (see *Updating the Offsets File* on page 74), then the values will be within 5% of the numbers reported by the Analysis Pipeline.

Tile Time: Measuring Software Overhead

The Tile Time metric shows the time spent imaging each tile, excluding the exposure time noted in the recipe.

Creating Reports and Viewing Data

Launching Run Browser

Automatically

During installation, Run Browser is configured to launch automatically after first-base incorporation.

Manually

If you wish to launch Run Browser manually at any point:

- Navigate to <install directory>\Illumina\RunBrowser_v06\bin\Release and double click RunBrowser.exe.

Changing Launch Settings

If you wish to change any of the launch settings, do the following:

1. Navigate to <install directory>\Illumina.
2. Open GalaxyRunConfig.xml in a text editor.
3. Edit the following parameters:

Setting	Description
`<RunBrowserExe>C:\Illumina\RunBrowser_v06\bin\Release\RunBrowser.exe</RunBrowserExe>`	Sets the path to the Run Browser executable.
`<EnableRunBrowserAutoLaunch>true</EnableRunBrowserAutoLaunch>`	Determines whether Run Browser launches automatically.
`<RunBrowserFirstCycleEvalOnly>true</RunBrowserFirstCycleEvalOnly>`	Determines whether Run Browser launches automatically only after first-base incorporation, or after first-base incorporation ***and*** after the completed run.

Running a Report

After performing autofocus calibration (see *Performing Autofocus Calibration* on page 69), run a First Cycle report to gather more information about the first-base incorporation and determine whether to complete the run.

1. If Run Browser does not open automatically on the dedicated instrument workstation, open it manually. For instructions, see *Launching Run Browser* on page 84.
2. Select **File | Open**.
3. Navigate to <install directory>\Illumina\DataCollection_v06\bin\Service\07-03-23_17-04-32, where the numbers represent YY-MM-DD_HH-MM-SS of the latest run.
4. Open the log files from the recent run (Control- or Shift-select to select more than one file).

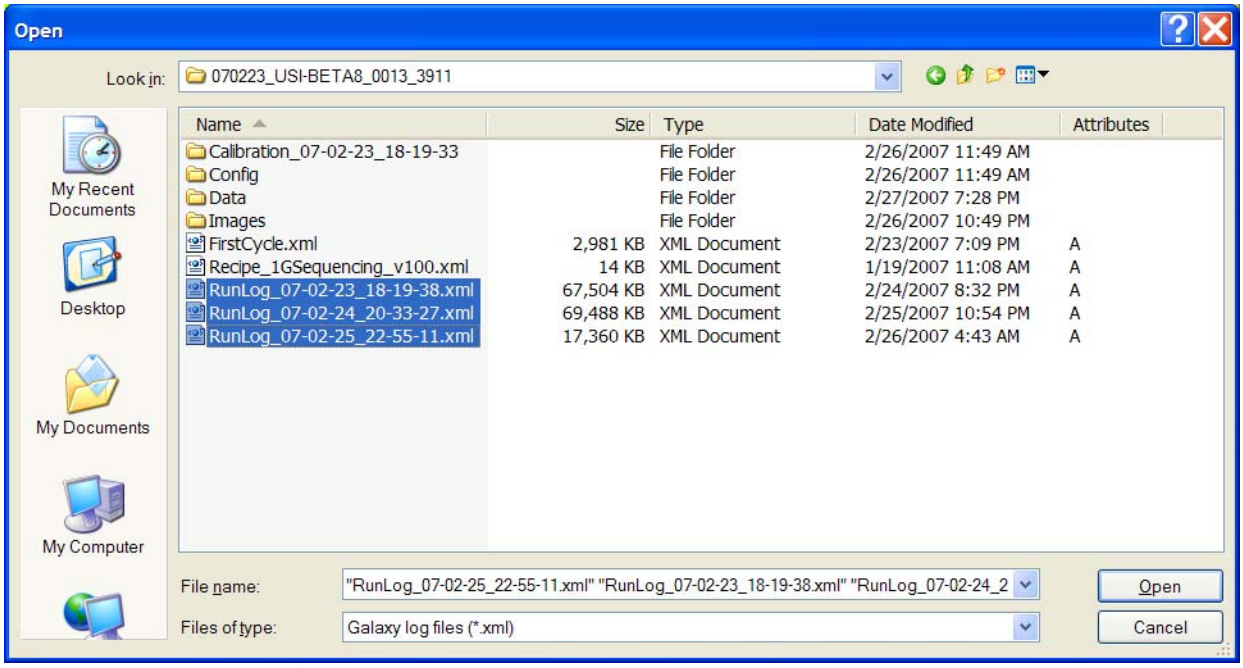

Figure 62 Open Log File

5. When prompted for the data location, select the <run folder>\Data\Goldcrest directory.

   It may take a few moments for the tiles to load.

CAUTION You should operate Run Browser on the dedicated instrument workstation, on log files that are stored locally. If you open log files that are stored on a network drive, it may take one or more hours for the files to open.

   The data from the run appear in the Flow Cell window, along with a map of the flow cell. You can check quite a bit of the data in this window by selecting different quality metrics and mousing over the flow cell to see values for each tile.

6. Select **View | Report**. The Report window opens.

Figure 63 Empty Report Window

7. To generate a First-Cycle report, click **First-Cycle Report** in the left sidebar.

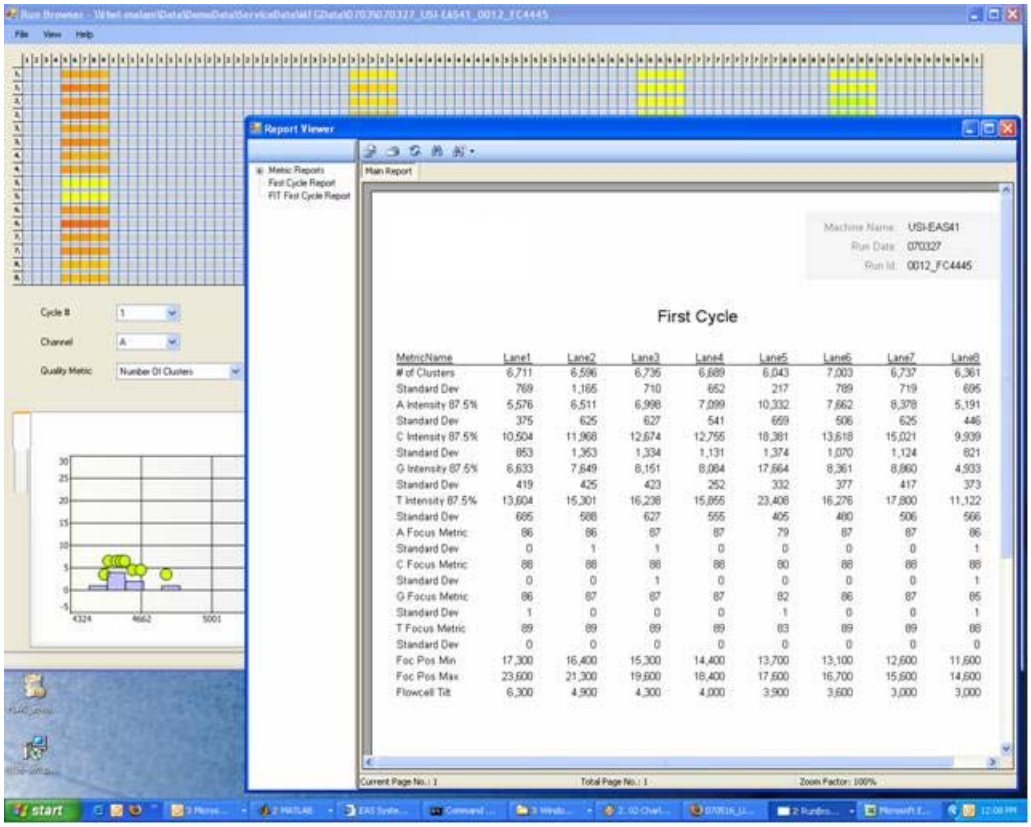

Figure 64 Sample First-Cycle Report

8. To generate a Metrics report:
   a. Click **Metric Reports** in the left sidebar.
   a. Expand one of the metrics, such as Cluster Intensity Median.
   b. Select the statistical operation with which to summarize the tiles in each lane of a cycle (Min, Max, Median, Mean, or SD).
   c. [Optional] If available, select one of the four image channels (A,C, G, T). This only applies to metrics that have different values for each channel.

   When you select a report, the report data appear in the right pane of the Report window.

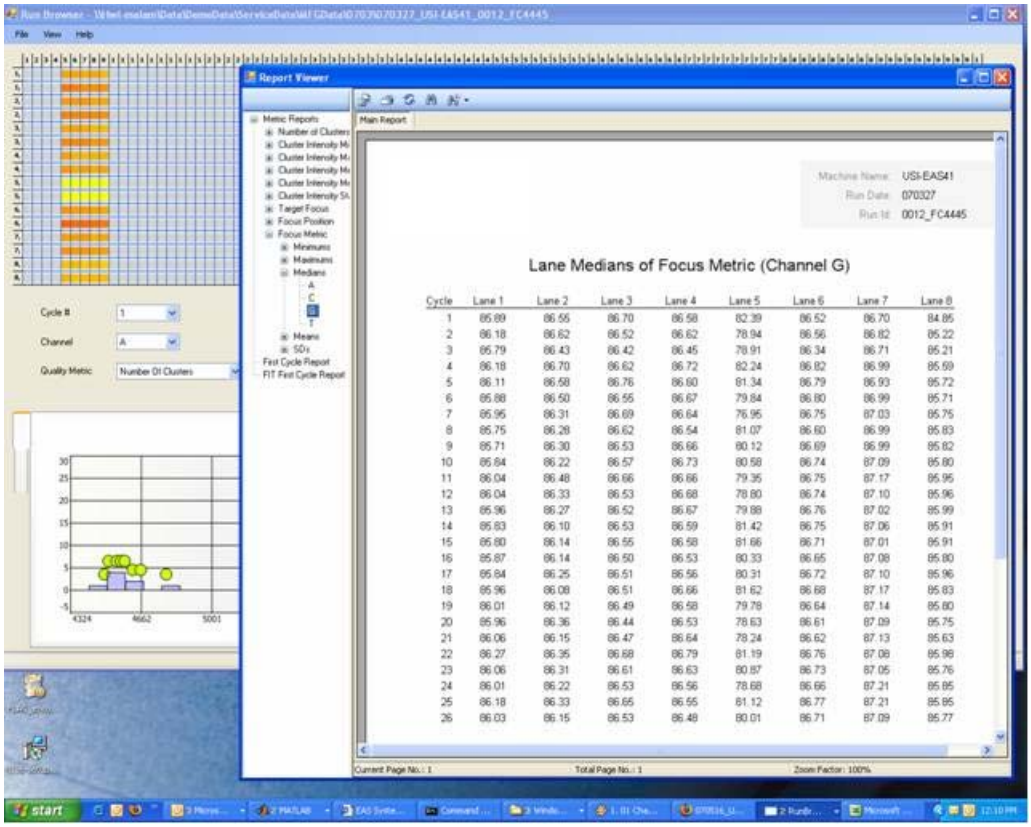

Figure 65 Sample Metric Report

9. Click a toolbar button to perform the associated action:

Table 15 Run Browser Report Viewer Buttons

Button	Function
	Export in one of the following formats: • Crystal Reports (*.rpt) • Portable Document Format (*.pdf) • Microsoft Excel (*.xls) • Microsoft Excel Data Only (*.xls) • Microsoft Word (*.doc) • Rich Text Format (*.rtf)
	Print report
	Refresh window
	Find text
	Zoom view (options provided in flyout menu)

Checking First Cycle Results in the Flow Cell Window

This section explains how to check some useful first-base incorporation metrics in the Flow Cell window. You should also generate a First-Cycle report to see a complete summary of the data.

1. Follow the instructions in *Running a Report* on page 85 to open the data from a first-base incorporation.
2. To check the tilt of the flow cell:
   a. Select Focus Position from the **Quality Metric** list box.
      The map of the flow cell changes to show focal positions at the front, middle, and rear of the flow cell.
   b. Ensure that **Cycle #** is set to 1.
      This value is the same for all channels, so you do not need to change the channel selection.
   c. Subtract the minimum focus stage level from the maximum. The difference should be less than 15,000 nm.
      To view the focal position of a given tile, hover the mouse over the tile in the flow cell map.

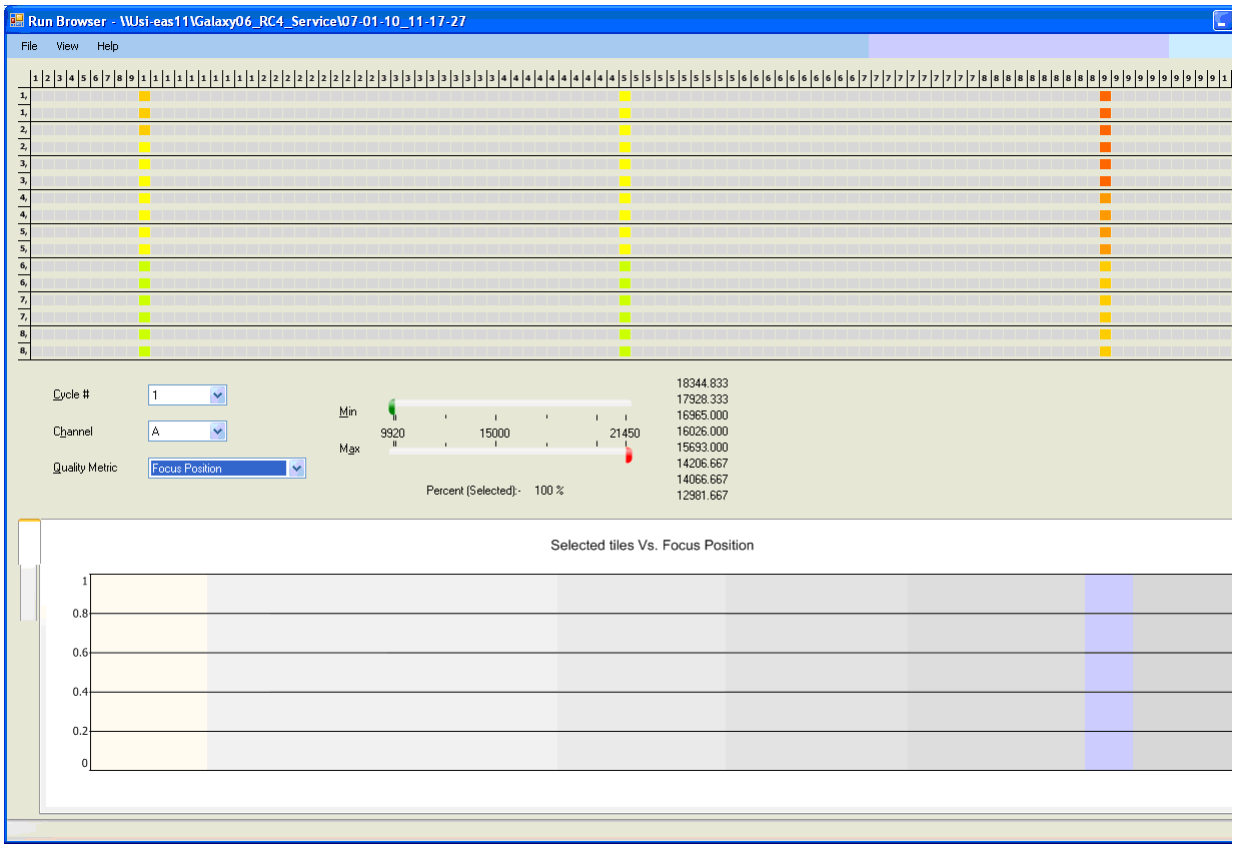

Figure 66 Focus Stage Level

3. To check the cluster intensity values:
   a. Select Cluster Intensity 87.5th %ile from the **Quality Metric** list box.
   b. Ensure that **Cycle #** is set to 1.
   c. Select each channel in turn from the **Channel** list box.

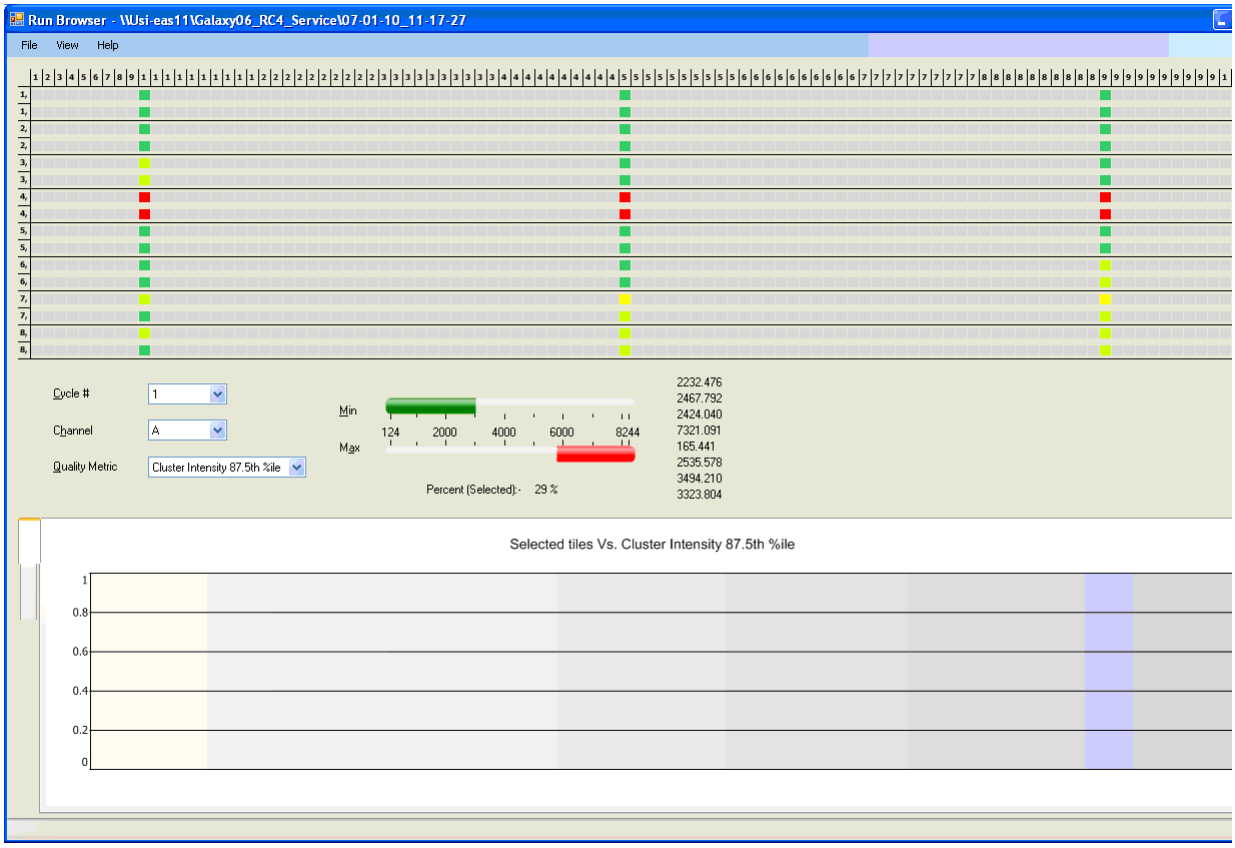

Figure 67 Cluster Intensity Levels

The average intensity of the selected tiles for each lane appears in a list to the right of the Min/Max bars.

d. Evaluate the intensity values for each channel according to the values in this table:

Table 16 Cluster Intensity Values at 87.5%ile

	A	C	G	T
High Confidence	> ,8,000	> 10,000	> 10,000	> 12,000
Reasonable Confidence	> 5,000	> 5,000	> 5,000	> 6,000
Low Confidence	< 3,000	< 3,000	< 3,000	< 5,000

4. To check the focal quality:

If you are running high density flow cells (clusters > 15,000), do not use this metric. The data will not be correct.

a. Select Focus Metric from the **Quality Metric** list box.
b. Ensure that **Cycle #** is set to 1.
c. Select each channel in turn from the **Channel** list box.

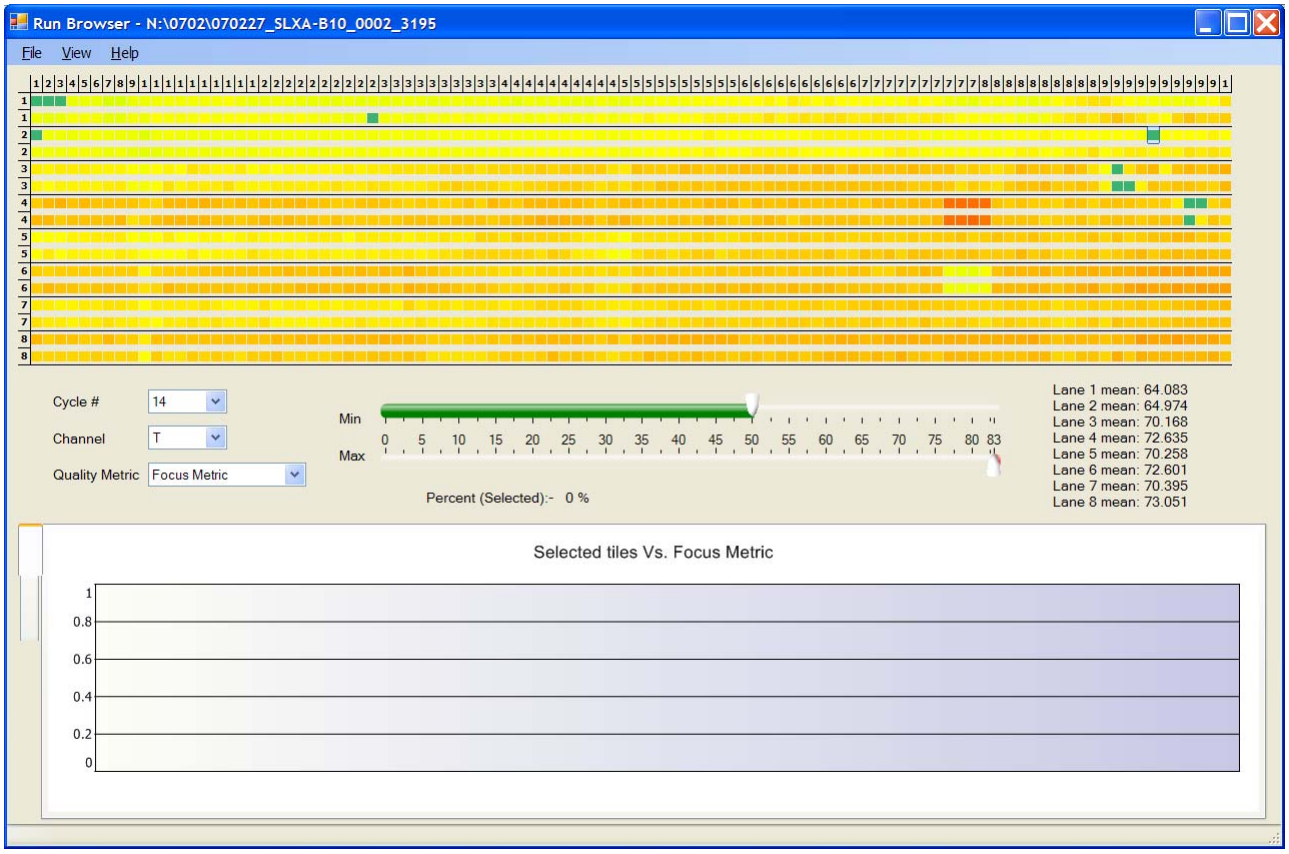

Figure 68 Run Browser Focus Metric

The average focal quality of the selected tiles for each lane appears in a list to the right of the Min/Max bars.

d. Ensure that the focus metric for each lane is greater than 60.

Appendix A
Run Folders

Topics

Introduction

Each run generates a run folder that contains data files and log files for all runs. All run folders are stored in a single folder (see *Run Folder Path* on page 94).

When you start a run, the system prompts you to enter the folder name for it. By default, the folder is named in this format:

YYMMDD_<Workstation Name>_<Run Number>

Example: 070320_WORKSTATION-487_0002

The run number increments by one each time you perform a run on a given workstation. Typically, users delete the run number and replace it with the flow cell ID. The name cannot have any spaces.

Run Folder Path

All run folders are stored in a single folder. The name and location of this folder are set in <install location>\bin\Config\RCMConfig, in this line:

```
<Run Path="D:\Runs" />
```

To change the run folder path, either change this line or select **Run | Select Run Folder Root** in the software. Enter the full path and folder name.

Contents of Run Folders

Table 17 Run Folder Contents

Subfolder	Key Files/Folders	Description
[Root level]	Configuration files	All active configuration files for this run are copied to the root of the run folder path.
	run.completed file	Appears in the folder when a run is successfully completed. You can tell whether a run is still in progress by checking for this file.
	Sample Sheet	If you create a sample sheet, it is copied to this location, and its name is added to the params file in this folder.
	Recipe_*.xml	When you start a run, a copy of the recipe is moved to the run folder. The name will be prefixed by "Recipe_". If you stop the run, open this recipe to start up again where you left off.

Table 17 Run Folder Contents

Subfolder	Key Files/Folders	Description
	*.params file <YYMMDD>_<workstation name>_<run number>.params	Identifies the instrument, the number of cycle exposures, and the name of the sample sheet.
	Log file RunLog_<date>_<time>.xml	Whenever you stop a run, a log file detailing the duration of every command appears in the root of the run folder path. If you stop and resume the run multiple times, there will be a separate log file for each session. Other log files for the run can be found in <install location>\bin\Config\LogFiles.
Images	Lane folders One folder per lane <Sample-ID>_L<Lane #> Lane folders contain one subfolder for each cycle	Contains TIFF files for each lane, named <sample>_<lane>_<pos>_<base>.tif
	Focus folders (optional)	Contains the TIFF focus images.
Data	Image Analysis folders One folder for each time a batch of images is processed by an image analysis package C<first cycle>-<last cycle>_<software><version>_<DD-MM-YYYY>_<user><.optional version number>	Usually contains the following file types, with one file per tile: Intensity—*_int.txt Sequence—*_seq.txt Quality Score—*_prb.txt
	.params file	Contains records pertaining to each image analysis subfolder. Enables you to browse the image analysis runs without having to go into each subfolder.
Calibration_<DateTime>	Calibration results and image files	

Appendix B

Sample Sheets

Topics

Introduction

The sample sheet contains information about the samples in each lane of the flow cell. This information can be used by the Cluster Station, Genome Analyzer, and data analysis tools. The content and format of the sample sheet are defined in a customizable schema file.

The sample sheet editor is only available once a network is set up.

There are two ways to create a sample sheet:

- Enter the data in the Sample Sheet Editor when you begin a run in the Cluster Station.
- Enter the data in the standalone Sample Sheet Editor.

Sample sheets are stored in a network location that is accessible to both the Cluster Station and the Genome Analyzer. After you create a sample sheet for a flow cell during cluster generation, you can access it later when you begin sequencing.

To Enter Sample Sheet Data in the Cluster Station

1. When you click **Start** to begin a run, the Cluster Station opens the Sample Information dialog box.
2. Fill in all of the boxes in the Editor.

The fields you see may be different from those that appear here, may require data to be in a certain format, or be optional. All of this is defined in the sample sheet schema file.

3. Click **OK**.
4. When prompted, confirm or change the folder storing the data for this run, and then click **OK**.

   If there is no sample sheet folder in the location defined in RCMConfig.xml, the software creates one. The sample sheet is automatically saved as <flow cell ID>.xml.
5. If the RCMConfig file requires it, then when you start a run on the Genome Analyzer, it will look for the sample sheet that matches the flow cell ID you entered. It validates the sheet against the schema, and saves a copy of the sample sheet in the local run folder.

   The data analysis tools use the copy of the sample sheet stored in the Genome Analysis run folder.

To Enter Sample Sheet Data in the Standalone Editor

1. Run the SampleSheetEditor.exe application in the <install location>bin\Release folder.
2. Fill in the data and click **OK**.

NOTE

The fields you see may be different from those that appear here, may require data to be in a certain format, or be optional. All of this is defined in the sample sheet schema file.

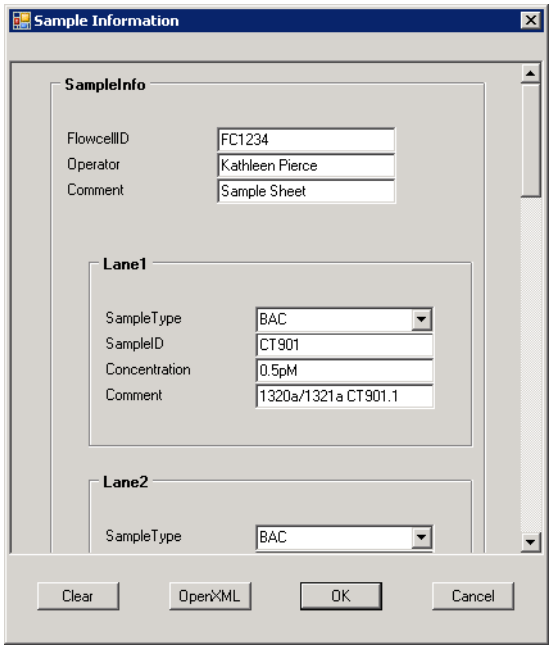

Figure 69 Sample Sheet Editor

3. Name the sample sheet file after the flow cell ID, with an *.xml extension: for example, FC3456.xml.
4. Save the sample sheet in the network location indicated in the RCMConfig.xml file. When prompted for a sample sheet in the Cluster Station or Genome Analyzer, navigate to the file.

Configuring Sample Sheet Behavior

The RCMConfig.xml file contains a line that defines the behavior of the sample sheet:

```
<SampleSheet SchemaFile="..\Config\SampleSheet.xsd"
SampleSheetPath="..\SampleSheets" Required="false"
Show ="false" />
```

- **SchemaFile**—The location of the sample sheet schema file (*.xsd). This must be a networked location that is accessible to both the Cluster Station and Genome Analyzer workstations. The Genome Analyzer validates sample sheets against the schema file.
- **SampleSheetPath**—The location of individual sample sheets. This must be a networked location that is accessible to both the Cluster Station and Genome Analyzer workstations.
- **Required**—Whether or not the Genome Analyzer requires a sample sheet.
- **Show**—Whether or not the Sample Editor dialog box appears.

Sample Sheet Example

This section shows the contents of an imaginary sample sheet file.

```
<?xml version="1.0" encoding="utf-8" ?>
<SampleInfo>
  <FlowcellID>FC5312</FlowcellID>
  <Operator>Lou</Operator>
  <Comment>BAC/MONO</Comment>
<Lane1>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane1>
<Lane2>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane2>
<Lane3>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
```

```
  <Concentration>0.5pm</Concentration>
  </Lane3>
<Lane4>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane4>
<Lane5>
  <SampleType>Monotemplate</SampleType>
  <SampleID>HCT031</SampleID>
  <Concentration>0.3pm</Concentration>
  </Lane5>
<Lane6>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane6>
<Lane7>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane7>
<Lane8>
  <SampleType>BACControl</SampleType>
  <SampleID>CT391</SampleID>
  <Concentration>0.5pm</Concentration>
  </Lane8>
  </SampleInfo>
```

Appendix C
Recipes

Topics

Introduction

Recipes are XML files containing a series of commands. To perform runs on the Cluster Station or the Genome Analyzer, you open and execute the appropriate recipe.

Each recipe file has two main sections: ChemistryDefinitions and Protocol.

- **ChemistryDefinitions**—Contains multiple named blocks which contain a sequence of chemistry commands. For example, some Cluster Station chemistry definitions are TemplateDNAHybridization, Blocking-CyclicPumping, and WashOneStepFullProtocolLines. Some Genome Analyzer chemistry definitions are Prime, Deblock, and FirstBase.
- **Protocol**—Invokes chemistry definitions in a particular sequence to perform the run. Genome Analyzer sequencing protocols perform image data acquisition steps (incorporation and cleavage) in addition to the chemistry. All protocols may contain UserWait messages, which pause the run and trigger dialog boxes with instructions for the user.

Genome Analyzer recipes also contain the following section:

- **Tile Selection**—Determines which rows and lanes are imaged during the incorporation and cleavage cycles. You can shorten the run by limiting the number of tiles imaged, although this generates concomitantly less data.
- **Incorporation**—The portion of the sequencing protocol where fluorescent bases are incorporated onto complementary bases in the clusters.
- **Cleavage**—The portion of the protocol following each incorporation cycle, where the fluorescent bases are washed from the clusters. Cleavage is also referred to as **Deblock**.

Stopping and Restarting a Recipe

Click **Stop** if you wish to stop the currently executing command. Some commands stop immediately, while others need to finish before stopping. Completed steps have a check mark beside them. When a run is stopped, the system places itself into a partial safe state. The partial safe state function executes a pump initialization.

At the beginning of a run, the active recipe file is automatically copied to the run folder. If the run stops before completion, there are two ways to restart the run from the point where it left off.

1. If you have closed the application, loaded another recipe, or otherwise navigated away from the run, select **File | Open Recipe**. Navigate to the run folder and open the recipe from there.
2. Do one of the following to select the restart point:
   - Highlight the first protocol step that does not have a check mark.
   - Highlight any *italicized* command, whether it is before or after the point where you left off.

   The **Resume** button becomes active when you select a valid restart point. If you stopped during an Incorporation or Cleavage imaging step, then the imaging cycle restarts from the first tile.

If an error occurs, then the run stops automatically and the instrument is placed in a safe state.

Protocol Section

Here is a snippet of a Genome Analyzer recipe file, showing part of the Protocol section. Cluster Station recipes, and other Genome Analyzer recipes, use different commands but have the same general format.

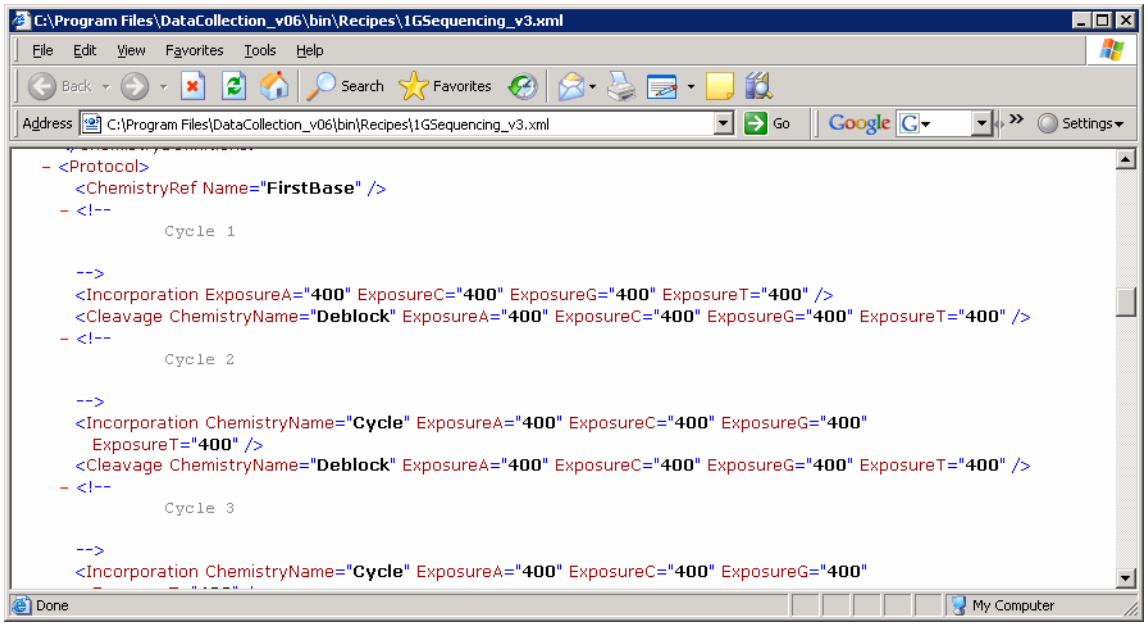

Figure 70 Protocol Section of Sequencing Recipe File

In Genome Analyzer recipes, you can specify identical chemistry steps in two ways:

- In separate lines of the protocol:

  ```
  <ChemistryRef Name=”FirstBase” />
  <Incorporation ExposureA=”400” ... / >
  ```

- Inside the line that invokes the incorporation or cleavage imaging cycle:

  ```
  <Incorporation ChemistryName=”FirstBase” Expo-
  sureA=”400” ... />
  ```

In the second example, the incorporation step includes both the incorporation chemistry and the imaging cycle.

Chemistry Definition Section

Here is a snippet of a Genome Analyzer recipe file, showing a chemistry definition. Cluster Station recipes, and other Genome Analyzer recipes, use different chemistry definitions but have the same general format.

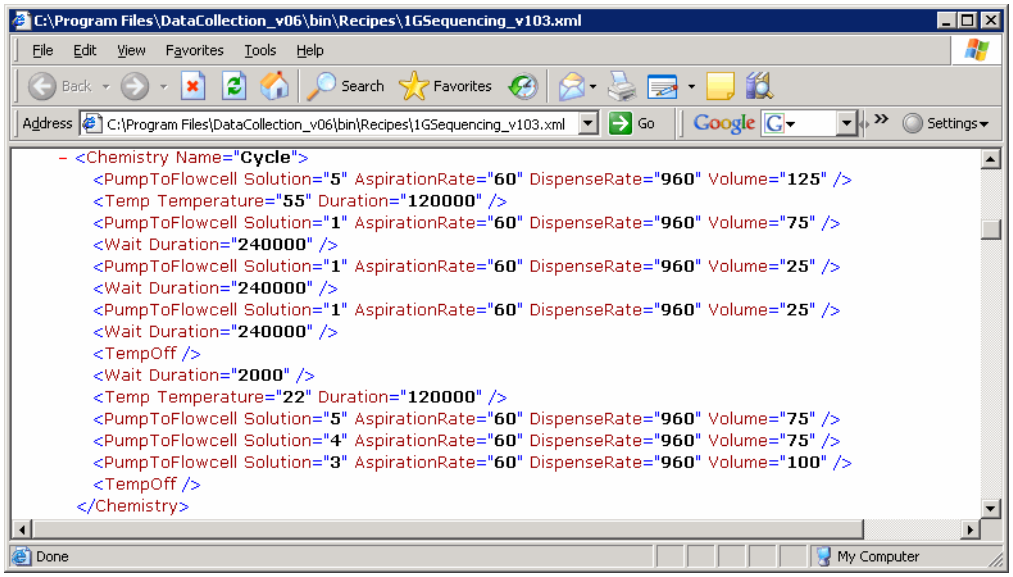

Figure 71 Chemistry Definition Section of Sequencing Recipe File

General Commands

These commands may appear in any recipe for the Cluster Station or Genome Analyzer.

Table 18 General Recipe Commands

Command	Mandatory Attributes	Action
TempSet	**Temperature**—Flow cell temperature in degrees Celsius. **Duration**—Length of time that the temperature will be maintained, in milliseconds.	Sets the flow cell temperature.
TempRamp	**Temperature**—Target flow cell temperature in degrees Celsius. **Rate**—The rate of change, in degrees per second.	Ramps the temperature of the flow cell.
Wait	**Duration**—The wait time, in milliseconds.	Sets the time for the chemistry step to complete.

Table 18 General Recipe Commands

Command	Mandatory Attributes	Action
UserWait	[Optional] **Message**—An explanatory or instructional message that appears in a dialog box when the run pauses.	Normally, this is used when the run requires user input to continue. The user can click **OK** to continue the run or **Cancel** to stop the run.

Cluster Station Commands

These commands appear only in Cluster Station recipes.

Table 19 Cluster Station Recipe Commands

Command	Mandatory Attributes	Action
Pump	**Reagent**—The number of the solution to be pumped. **AspirationRate**—Pump rate in µl/minute. **Volume**—Pump volume in µl.	Pumps reagent to the flow cell. The dispense rate is fixed at 3000 µl/minute.
Prime	**Reagent**—The number of the reagent to be primed.	Primes the lines. Each prime command pumps 20 µl of reagent.
TempSet	**Temperature**—Flow cell temperature in degrees Celsius. **Duration**—Length of time that the temperature will be maintained, in milliseconds.	Sets the flow cell temperature.
TempRamp	**Temperature**—Target flow cell temperature in degrees Celsius. **Rate**—The rate of change, in degrees per second.	Ramps the temperature of the flow cell.

Genome Analyzer Commands

These commands appear only in Genome Analyzer recipes.

Table 20 Genome Analyzer Recipe Commands

Command	Mandatory Attributes	Action
PumpToFlowcell	**Solution**—The number of the solution to be pumped. **AspirationRate**—Pump rate in µl/minute. Default is 60; maximum value is 120. **DispenseRate**—Pump rate in µl/minute. Default is 2000. **Volume**—Pump volume in µl.	Pumps reagent from the named solution bottle/tube through the flow cell.
Incorporation	[Optional] **ChemistryName**—Invokes a particular chemistry as part of the incorporation cycle, such as FirstBase. **ExposureA**, **ExposureG**, **ExposureC**, and **ExposureT**—The image exposure time for each image channel during the incorporation cycle, in milliseconds.	Sets the exposure time for each image channel during incorporation. May also invoke incorporation chemistry.
Cleavage	[Optional] **ChemistryName**—Invokes a particular chemistry as part of the cleavate cycle, such as Deblock. **ExposureA**, **ExposureG**, **ExposureC**, and **ExposureT**—The image exposure time for each image channel during the cleavage cycle, in milliseconds.	Sets the exposure time for each image channel during cleavage. May also invoke cleavage chemistry. You do not have to include the Cleavage command in the protocol. If you do not, then you must explicitly call out ChemistryRef="Cleavage" to perform the cleavage chemistry.
RowRange	**Min**—The first row in the range {1–100} **Max**—The last row in the range {1–100}	Identifies a range of adjacent rows for incorporation or cleavage.
LaneRange	**Min**—The first lane in the range {1–4} **Max**—The last lane in the range {1–4}	Identifies a range of adjacent lanes for incorporation or cleavage. LaneRange only applies to the first four lanes.
Lane	**Index**—The number of the lane for incorporation or cleavage	Identifies one or more lanes for incorporation or cleavage. Write separate <Lane Index= ...> commands for each lane. You can include <Row> or <RowRange> commands inside the <Lane> command to limit the number of rows within each lane.
Row	**Index**—The number of the row for incorporation or cleavage	Identifies one or more rows for incorporation or cleavage. Write separate <Row Index= ...> commands for each lane.

Service Recipes

Service recipes are protocols for maintaining the flow cell and instrument. To make a recipe a service recipe, add the Type attribute to the RecipeFile element as follows:

```
<RecipeFile Type="Service">
```

To specify a normal recipe, add the following:

```
<RecipeFile Type="Normal">
```

A service recipe causes the software to skip the sample sheet dialog box and to suppress the run folder dialog box. All information relating to the run, such as the recipe and config files, is saved in the following folder:

..\Service, where the parent folder is
<install directory>\DataCollection_v<#>\bin

The folder containing the information from this run is named for the date and time that the recipe was executed.

The first-base incorporation recipe is a service recipe.

User-Defined Recipes

You can create and edit recipes in a text editor such as Notepad. User-defined recipes are validated upon loading.

Cluster Station recipes must meet two requirements to be considered valid. First, a recipe must have fewer than 20 temperature set points. Second, a recipe cannot contain any sequential repeating pattern of two or more temperature set points.

Configuring Tile Selection

You can edit Genome Analyzer sequencing recipes to select only certain tiles for imaging.

Exercise extreme care when editing a recipe. Always make a backup copy of the recipe before altering it.

By default, all lanes and rows will be imaged, which maximizes the amount of data captured for each experiment. However, in some cases you might want to shorten the run by reducing the area to be imaged. To narrow the number of lanes or rows for an experiment, use the Row, Lane, RowRange, and LaneRange tags.

Reducing the Number of Rows

This selection chooses rows 6–10, 21–25, 36–40, etc. for Incorporation. All lanes within those rows are selected by default, unless the LaneRange command is used to reduce the number. For cleavage, this selection lists specific rows to image; as with incorporation, every lane is selected by default.

```
<TileSelection>
    <Incorporation>
      <RowRange Min="6"  Max="10" />
      <RowRange Min="21" Max="25" />
      <RowRange Min="36" Max="40" />
      <RowRange Min="51" Max="55" />
      <RowRange Min="66" Max="70" />
    </Incorporation>
    <Cleavage>
      <Row Index="10" />
      <Row Index="35" />
      <Row Index="55" />
    </Cleavage>
</TileSelection>
```

Reducing the Number of Lanes

This selection chooses only the middle four lanes for incorporation imaging. For cleavage imaging, only three rows in Lane 1 are selected.

```
<TileSelection>
    <Incorporation>
      <Lane Index="3"/>
      <Lane Index="4"/>
      <Lane Index="5"/>
      <Lane Index="6"/>
    </Incorporation>
    <Cleavage>
      <Lane Index="1">
        <Row Index="10"/>
        <Row Index="35"/>
        <Row Index="70"/>
      </Lane>
```

```
    </Cleavage>
</TileSelection>
```

Sample Genome Analyzer Recipe with Annotations

This section examines the structure of a recipe, using a Genome Analyzer sequencing recipe as an example. Cluster Station recipes and other Genome Analyzer recipes have different content, but similar sections, commands, and attributes.

Tile Selection

```
<TileSelection>
```

The Tile Selection portion of the recipe indicates the specific tiles to be imaged. Only a subset of the tiles are imaged during first-base incorporation, while all tiles are imaged during the complete sequencing run.

Comment

```
<!--
  <Incorporation />  This results in number of lanes
         equal 0 => do all of them
  <Lane />  Not legal, don't know which lane is
         being referenced
  <Rows />  or leaving out the 'Rows' element has
         the same effect; the number of rows is 0 =>
         do all of them.
    -->
```

<!-- xxxxxx -->This notation allows for a comment to be inserted. This character string tells the software not to interpret the comments as a command.

Comments provide instructional notation about the recipe.

Incorporation

```
<Incorporation>
```

The Incorporation section of the recipe refers to images taken after nucleotide incorporation occurs.

```
<Lane Index="1">
```

By specifying the Lane Index you can modify the number of tiles to be imaged per channel.

```
<RowRange Min="23" Max="27" />
<RowRange Min="33" Max="37" />
<RowRange Min="43" Max="47" />
<RowRange Min="53" Max="57" />
```

Tiles 23–27, 33–37, 43–47, 53–57 from Lane 1 will be imaged.

RowRange Min="23" Max="27" indicates five rows will be imaged. This command is inclusive; that is, 23, 24, 25, 26, and 27 will be imaged.

```
    </Lane>
    <Lane Index="2">
      <RowRange Min="23" Max="27" />
      <RowRange Min="33" Max="37" />
      <RowRange Min="43" Max="47" />
      <RowRange Min="53" Max="57" />
```

Tiles 23–27, 33–37, 43–47, 53–57 from Lane 2 will be imaged.

```
    </Lane>
    <Lane Index="4">
      <RowRange Min="33" Max="37" />
      <RowRange Min="53" Max="57" />
```

In Lane 4, however, only 10 rows will be imaged.

```
    </Lane>
  </Incorporation>
```

End of Incorporation section.

An easy way to image the same tiles per channel is as follows:

```
  <Incorporation>
    <Row Range Min="1" Max "75">
  </Incorporation>
```

Cleavage

```
  <Cleavage>
```

The Cleavage section defines the tiles to be imaged once the fluor is removed. These images can be used to double-check the efficiency of the cleavage reaction. A small number of images are taken past the cleavage step (typically three per channel).

```
    <Lane Index="1">
      <Row Index="10" />
      <Row Index="35" />
      <Row Index="70" />
```

Tiles 10, 35, and 70 will be imaged.

Row 10 is near the front of the flowcell, Row 35 is near the middle, and Row 70 is near the back.

```
    </Lane>
    <Lane Index="2">
      <Row Index="10" />
      <Row Index="35" />
      <Row Index="70" />
    </Lane>
    <Lane Index="7">
```

```
            <Row Index="10" />
            <Row Index="35" />
            <Row Index="70" />
          </Lane>
          <Lane Index="8">
            <Row Index="10" />
            <Row Index="35" />
            <Row Index="70" />
          </Lane>
        </Cleavage>
```

End Cleavage.

```
    </TileSelection>
```

End Tile Selection.

Chemistry Definitions

```
    <ChemistryDefinitions>
```

In <ChemistryDefinitions>, a series of operations can be grouped together and defined as a "chemistry." A chemistry consists of one or more commands and can include temperature changes, reagent deliveries, wait steps, and user messages.

Volumes are in milliliters.

Waits are in milliseconds.

These chemistries are later called during the Protocol portion of the recipe.

```
        <Chemistry Name="Prime">
        </Chemistry>
        <Chemistry Name="End">
```

Define protocol: End last stem.

```
          <PumpToFlowcell Solution="2"
               AspirationRate="60" DispenseRate="2000"
               Volume="4000" />
```

Pump 4 ml of solution 2 (H_20).

```
        </Chemistry>
```

First Base Protocol

```
        <Chemistry Name="FirstBase">
```

Define First Base protocol.

```
          <PumpToFlowcell Solution="5"
               AspirationRate="60" DispenseRate="2000"
               Volume="1000" />
```

Pump 1 ml of Incorporation Buffer.

```
          <Temp Temperature="55" Duration="120000" />
```

Set temperature to 55°C wait 2 minutes.

```
<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of incorporation mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />
```

Pump 200 µl of incorporation mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />
```

Pump 200 µl of incorporation mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<Temp Temperature="22" Duration="120000" />
```

Set temperature to 22°C, wait 2 minutes.

```
<PumpToFlowcell Solution="5"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of Incorporation Buffer.

```
<PumpToFlowcell Solution="4"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of High Salt Buffer.

```
<PumpToFlowcell Solution="3"
     AspirationRate="60" DispenseRate="2000"
     Volume="800" />
```

Pump 800 µl of Scan Buffer.

```
<TempOff />
```

Temperature off.

```
<UserWait Message="First Base Incorporation
     Chemistry Complete
     To perform manual focus and 1st base
     evaluation now, press cancel" />
```

User wait step.

```
</Chemistry>
```

End First Base definition.

Deblock Protocol

```
<Chemistry Name="Deblock">
```

Define Deblock (cleavage) protocol.

```
<PumpToFlowcell Solution="7"
     AspirationRate="60" DispenseRate="2000"
     Volume="1000" />
```

Pump 1 ml of Cleavage Buffer.

```
<Temp Temperature="55" Duration="120000" />
```

Set temperature to 55°C, wait 2 minutes.

```
<PumpToFlowcell Solution="6"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of Cleavage Mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<PumpToFlowcell Solution="6"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />
```

Pump 200 µl of Cleavage Mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<PumpToFlowcell Solution="6"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />
```

Pump 200 µl Cleavage Mix.

```
<Wait Duration="240000" />
```

Wait 4 minutes.

```
<Temp Temperature="22" Duration="120000" />
```

Set temperature to 22°C, wait 2 minutes.

```
<PumpToFlowcell Solution="5"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of Incorporation Buffer.

```
<PumpToFlowcell Solution="4"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Pump 600 µl of High Salt Buffer.

```
<PumpToFlowcell Solution="3"
     AspirationRate="60" DispenseRate="2000"
     Volume="800" />
```

Pump 800 µl of Scan Buffer.

```
<TempOff />
</Chemistry>
```

End Deblock definition.

Cycle Definition

```
<Chemistry Name="Cycle">
```

"Cycle" is just like First Base, but without a User Wait.

In the Cycle chemistry, reagents and temperatures are controlled for the incorporation of the nucleotides and to make the cluster environment amenable for imaging.

```
<PumpToFlowcell Solution="5"
     AspirationRate="60" DispenseRate="2000"
     Volume="1000" />
```

Pump Incorporation Buffer.

```
<Temp Temperature="55" Duration="120000" />
```

Set temperature to 55°C.

```
<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Incorporation Mix is delivered in a "pulsed" fashion. It is delivered and then sits for 4 minutes in the flow cell.

```
<Wait Duration="240000" />

<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />

<Wait Duration="240000" />

<PumpToFlowcell Solution="1"
     AspirationRate="60" DispenseRate="2000"
     Volume="200" />

<Wait Duration="240000" />

<Temp Temperature="22" Duration="120000" />
```

Ramp flow cell temperature to 22°C in preparation for imaging.

```
<PumpToFlowcell Solution="5"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />
```

Exchange Incorporation Buffer, then 5xSSC, and finally Scan Buffer.

```
<PumpToFlowcell Solution="4"
     AspirationRate="60" DispenseRate="2000"
     Volume="600" />

<PumpToFlowcell Solution="3"
     AspirationRate="60" DispenseRate="2000"
     Volume="800" />

<TempOff />

</Chemistry>
```

End Cycle definition.

```
</ChemistryDefinitions>
```

End Chemistry Definitions

Protocol

```
<Protocol>
```

In the Protocol section of the recipe, all of the recipe components defined to this point are combined to define instrument cycles of chemistries such as priming, washing, cleavage, and incorporation. The exact combination is determined by the instrument and the recipe.

This sample protocol from the Genome Analyzer consists of 26 cycles of incorporation followed by imaging. The Incorporation command takes two inputs, an optional chemistry and exposure times for each filter/base.

```
<ChemistryRef Name="FirstBase" />
```

Call up First Base protocol.

```
<!--      Cycle 1      -->
```

This is a comment indicating the following commands begin Cycle 1.

```
<Incorporation ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
```

Image incorporation tiles, exposure time: 400 ms per picture.

```
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
```

Call up Deblock chemistry image cleavage tiles: 400 ms each.

```
<!--      Cycle 2       -->
```

This is a comment indicating the following commands begin Cycle 2.

```
<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
```

Call up Cycle protocol image incorporation tiles.

```
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
```

Image deblock tiles.

```
<!--      Cycle 3      -->
```

Repeat.

```
<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />

<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />

<!--      Cycle 4       -->

<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
```

```
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
```

Cycle 4 consists of the Cycle chemistry followed by imaging of all tiles specified in the Incorporation section of Tile Selection at 400 ms per base. Once imaging is complete, cleavage occurs using the Deblock chemistry and then imaging of the tiles specified in the Cleavage section of the recipe.

```
<!--      Cycle 25       -->
<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
<!--     Cycle 26     -->
<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
<!--     Cycle 27      -->
<Incorporation ChemistryName="Cycle"
       ExposureA="400" ExposureC="400"
       ExposureG="400" ExposureT="400" />
<Cleavage ChemistryName="Deblock" ExposureA="400"
       ExposureC="400" ExposureG="400"
       ExposureT="400" />
<ChemistryRef Name="End" />
```

Call up End chemistry, after the last incorporation cycle, which delivers water from position 2.

```
</Protocol>
```

End Protocol.

Appendix D
Frequently Asked Questions

Topics

General

Do we need to optimize the Cluster Station and Genome Analyzer to ensure correct, consistent results?

No. The machines are standardized and will remain stable if left in the original configuration.

What are your recommendations for optimizing machine use?

As a best practice, you should have a number of flow cells in the 4°C refrigerator that are ready for primer hybridization and sequencing. This helps ensure that you can run the Genome Analyzer nearly continuously, maximizing its use. Backup flow cells also provide insurance against the failure of any one flow cell. If a flow cell fails first-base incorporation, you can simply run a backup flow cell and not waste the Genome Analyzer reagents or the machine time. To support this flexibility, the Cluster Station must be available for primer hybridization when needed.

How long can flow cells be stored?

After amplification, you can store flow cells indefinitely at 4°C. It then takes approximately 3 hours to linearize, block, and hybridize the primers before sequencing.

After blocking, you can store flow cells for one month at 4°C. It then takes approximately one hour to hybridize the primers before sequencing.

After primer hybridization, the sequencing should proceed as soon as possible. Illumina recommends you begin sequencing within four hours.

What is a recipe?

A recipe is a protocol for either the Cluster Station or the Genome Analyzer. Most of the standard recipes come preloaded on the instrument. For more information, see Appendix C, *Recipes*.

Can I edit recipes to change the chemistry, the number of cycles, etc?

You can edit recipes, but Illumina does not recommend that you do so, and cannot provide support for non-standard recipes. If you want to create a customized recipe, contact Illumina Technical Support or your Field Application Scientist.

Sample Prep

How many samples may be run at one time?

Flow cells are single-use, and all eight lanes must be used at the same time. They may be used for the same sample (common for sequencing applications), or for eight different samples (more common for RNA applications). There is a single flow cell per Cluster Sta-

tion Kit. There are no cross-contamination issues when using the Cluster Station or Genome Analyzer. However we recommend running one sample per prep gel during sample prep to avoid contamination. One lane should be used for the control. See *Controls* on page 126.

How long does it take to prepare samples?

It takes about one day to prepare genomic DNA for sequencing, three days to prepare RNA for gene expression analysis, and four days to prepare small RNA.

How many different libraries can we run on one flow cell?

You can run up to eight, not including indexing.

How much genomic DNA is needed for the genomic DNA prep kit?

1 µg of genomic DNA.

How is the DNA fragmented?

Illumina recommends using a nebulizer. Customers have also had success with sonication.

What are the differences between nebulization and sonication?

Nebulization leads to a tighter size distribution of DNA fragments than sonication but also leads to greater loss of sample.

How long does a prepared sample remain stable?

A prepared sample will remain stable indefinitely when stored at -20°C.

What is the optimal fragment size for the genomic DNA sample prep protocol?

The optimal fragment size is 120–170 bp.

What is involved in sample prep for small RNA applications?

To use small RNA as an example, start with 10 µg total RNA, cut out 18–30 bp using a ladder to estimate size, and elute from the gel. Ligate RNA adaptors and repeat gel purification to remove unligated adaptors. Perform cDNA synthesis, again with gel purification. The final step is a PCR step that could be run out on a gel. For more information, see *Preparing Samples for Analysis of Small RNA*, part # 11251913.

Is there a QC process for sample prep?

You can run a gel or sequence the product, if desired, to test the sample prep. There is currently no QC process for testing the product from the Cluster Station.

Cluster Station

How can I prevent cross-contamination?

Be especially careful when pipetting multiple samples into a strip tube, and change tips between each dispense. Once the samples are in the Cluster Station, the chance of cross-contamination is negligible.

Do you offer a single-cell protocol if people want to do a smaller experiment?

No, not at this time.

What is the variation for reagent delivery by the Cluster Station?

The Cluster Station has little variation. The most common causes of variation are due to poor attachment of the amplification or hybridization manifolds and air trapped in reagent lines or valves.

Can the heating element on the Cluster Station perform PCR?

No. This ability has been disabled.

The user guide says not to use the manual controls on the Cluster Station software. If we're not supposed to use them, why are they there?

The Cluster Station manual controls are mainly used for troubleshooting purposes. If you contact Illumina Technical Support, the support scientist may ask you to adjust some settings manually.

Clusters

How long does it take to generate clusters on a flow cell?

It takes about 5 hours on a Cluster Station to prepare a flow cell for sequencing, plus 1–2 hours for reagent preparation.

What happens if I increase the number of cycles on the Cluster Station?

The clusters become proportionately larger.

Is there a benefit to adding cycles so that I get larger clusters?

No. Over-large clusters are more likely to touch each other. Because the Genome Analyzer does not read clusters that touch each other, over-large clusters can actually reduce the amount of data collected. The Analysis Pipeline also ignores clusters over a certain size, so over-large clusters can be lost through filtering.

How can I optimize the clusters?

Short fragments tend to create tight, dense clusters. The optimal fragment size is 120–170 bp.

When can we determine the cluster density?

Cluster density can be determined after performing the first-base incorporation cycle on the Genome Analyzer. Generating a First-Cycle report from Run Browser will give you an estimation of the cluster brightness and number. For more information, see Chapter 5, *Run Browser Reports*.

Can SybrGreen be used to determine cluster density instead of first-base incorporation?

You can use SybrGreen to determine cluster density but it requires a fluorescent microscope to examine the flow cell before running the Genome Analyzer.

Amplification

What is the DNA concentration going into the amplification step?

This varies and should be optimized for each application and library template. For the control BAC, we recommend 5 pM final concentration. This yields about 20,000 clusters per tile.

What volume of DNA is pumped into each lane?

A total of 85 µl. The Cluster Station initially pumps in 75 µl, and then pumps 10 µl more to push out the air bubbles.

What if I don't have enough template DNA?

Template DNA concentration must be optimized at the sample preparation step.

Does cDNA work the same way?

Yes. cDNA feeds into the genomic DNA prep kit, at which point the two protocols are the same.

Can I walk away during the amplification temperature ramp (from 95°C to 40°C)?

The Cluster Station is not ready for walk-away automation until after you have attached the amplification manifold and checked for good flow through all channels.

Fluidics

When you perform a wash on the Cluster Station, how do you know which solution is flushed through the flow cell at any given time?

Hover your mouse over the highlighted recipe step in the software. A popup shows the instruction that the Cluster Station is currently executing.

Is it normal to see bubbles coming out of the flow cell on the Cluster Station?

It is normal during amplification. The heating and cooling naturally cause small bubbles to form and flow out through the hybridization manifold. There is no adverse effect on the assay.

You should not see large numbers of bubbles during any other process on the Cluster Station.

Genome Analyzer

How long does a sequencing run take?

On the Genome Analyzer, this depends on the number of cycles, which in turn depends on the application. GEX uses 18 cycles, small RNA uses 18–26 cycles, and sequencing uses up to 36 cycles. Each cycle takes 2.5 hours, assuming that you image all 200 tiles per lane.

Sample preparation and clustering take additional time before the run. After the run, the data analysis time depends on the available computing resources.

Controls

Illumina recommends running a control BAC or PhiX sample in one lane of each flow cell. Why do we need to give up a lane for an Illumina sample?

The data from the control are used to generate the matrix file. The analysis tool uses the control to calculate phasing/pre-phasing from this sample, and the relative proportion of the different bases. Without a control lane, the software would assume that the base composition of the sample is strictly balanced. While this is true of a total human genome, it might not be true of non-human genomes or a focused region of the human genome. Therefore, the control is necessary for all expression studies, small RNA studies, and reduced complexity studies.

Can we use the control for troubleshooting?

Yes, you can use the control library for troubleshooting purposes.

How do we tell the software what lane has the Illumina control?

This is determined during the setup of the Analysis Pipeline, a data analysis tool that runs on a separate server.

Once you know you have a good matrix, why do you need to keep using an Illumina control each time?

The normal variation between runs means that one matrix cannot apply universally.

How do I order the Illumina BAC or PhiX sample?

To order your Illumina BAC or PhiX sample, contact Illumina Technical Support.

What is a matrix file?

The matrix file accounts for cross talk between dyes. It is used for base calling.

What is deconvolution?

The ability to distinguish between two or more clusters that are in close proximity to each other.

What is the offsets file?

The offsets file is automatically generated during each run to account for the misalignment of images between the four dyes. The location of any given cluster shifts slightly depending on which filter you are using. Without a proper offset file, the cluster might be counted up to four times.

If adjustment to the Genome Analyzer optical system is made, it is necessary to recalculate the default offset file for the following run.

Software

During a cycle, the software suddenly turned off and restarted. What happened? Are my data safe? Do I need to restart the run?

Your data are safe and you do not need to restart the run. The shutoff and restart are in response to memory management issues in Windows, and occur at the beginning of a cycle. The software will automatically restart in about 30 seconds and pick up the recipe at the point it left off. While disconcerting, this is perfectly safe and prevents you from receiving an "out of memory" error during the run.

What is the best way to transfer image files from the Genome Analyzer computer to a network location?

Robocopy is a script that copies files from the local drive to a network location while the run is proceeding. This saves a considerable amount of time transferring data after completion of a run. Robomove copies files one at a time like Robocopy, performs a compare to ensure the integrity of the files, and then deletes them from the local drive.

Can I manually copy files to another location while the Genome Analyzer is running?

No. Robocopy is optimized to copy files during the chemistry cycles so that it does not interfere with image acquisition. If you try to copy, move, or delete files during a run, it can interfere with writing to the disk during image acquisition and cause an error.

How do I turn Robocopy or Robomove on or off?

This is done by modifying statements in a configuration file. To change the setting, contact your Illumina Technical Support, Field Application Scientist, or Field Service Engineer.

What happens if a Genome Analyzer run is interrupted before it completes? Can I restart?

It is possible to restart a run from where it was interrupted. The easiest way to do this is to load the recipe file from the last round of imaging and restart the run from where it left off. For more information, see *Stopping and Restarting a Recipe* on page 105.

However, if the cleavage step has started and you resume from the incorporation imaging step, you might overwrite incorporation images with cleavage images.

If the runs stops during imaging, pump some fresh solution # 3 (Scan Mix) through the flow cell, reload the recipe from the run folder, and resume from the interrupted round of images.

If the run stops during a chemistry step, reload the recipe from the run folder and resume from the first pump solution line.

What recipes do I use for the Genome Analyzer?

For the first round of chemistry, use GenomeAnalyzerbase1.xml. For the remaining rounds use GenomeAnalyzersequencing.xml.

How do I change the number of sequencing cycles?

To change the number of sequencing cycles, edit the GenomeAnalyzersequencing.xml file. For instructions, see Appendix C, *Recipes*.

The run finishes with a cycle chemistry. If adding more cycles, add the deblock chemistry for the last cycle.

Focus

Why do you have to manually focus if each image is autofocused?

Manual focusing sets the appropriate range and calibration curve for the autofocus algorithm. For more information, see *Adjusting Focus* on page 62.

What are the laser settings for manual focus?

Green laser on with T filter selected, 400 ms exposure.

Should I "home" the Genome Analyzer stage before each run?

No, it is not necessary. The stage is homed automatically during initialization. For more information, see *Adjusting Focus* on page 62.

Flow Cells

What is a tile?

A tile is an imaginary square within a lane, measuring 350 µm on each side. Each of the eight lanes in a flow cell is subdivided into multiple columns of 100 tiles. Every image is of one tile, so a tile is essentially the same thing as an image. Each tile is imaged five times per cycle, once for each base plus a focus image. Imaging fewer tiles is faster but does not capture all available data.

What is the difference between a lane and a channel?

A lane and a channel are the same thing. Channel can also refer to an image channel on the Genome Analyzer (A, C, G, or T).

Do I have to image every tile in a lane?

You do not have to image every tile, but typically you will want to do so to maximize the data generated from each lane. Imaging fewer tiles saves time in image capturing but not in chemistry.

How do I image fewer than the maximum number of tiles?

Note: Illumina does not recommend that you change the number of tiles being imaged during sequencing runs. The primary purpose of this flexibility is to assist support scientists during validation.

To change the number of columns imaged, open the tilelayout.xml file and change the column value to 1, 2, or 3. Any other options will generate an error.

To change the number of tiles imaged, see *Configuring Tile Selection* on page 110.

After the first-base incorporation, how do I tell if the flow cell is good and I should continue with the sequencing protocol?

See *Checking Quality Metrics* on page 69 and Chapter 5, *Run Browser Reports*.

Fluidics

Do I need to have a flow cell in place when I am running the fluidics?

In the Genome Analyzer, you absolutely need to have a flow cell in place. Failure to load a flow cell will introduce air into the system which will then need to be cleared before starting a run.

Fluidics can be tested in the Cluster Station using a washing bridge or a flow cell.

How do I manually test for leaks?

See *Checking for Leaks and Proper Reagent Delivery* on page 58.

What is the maximum flow rate for the syringes on the Genome Analyzer and the Cluster Station?

The flow (aspiration) rate used in the recipes is 60. The maximum rate is 120. Don't use more than 60 on the sequencer or it will cavitate and introduce a lot of bubbles in the system.

How do I check for even flow across the flow cell?

Start pumping solution # 5 (Incorporation Buffer), quickly lift the manifold, and immediately lower it while keeping the flow cell in place with a finger. Afterwards, clean the flow cell surface with methanol.

Some lanes in the flow cell are running backwards and there are lots of bubbles. What is going on?

This is probably due to a blockage in the front or rear manifold or in the input line leading to the front manifold. First, try re-seating the flow cell. If this doesn't work, try washing the lines with an aspiration rate of 120 µl per minute rather than the usual 60 to force the bubbles out.

What is the maximum volume for the syringes in the Genome Analyzer?

The maximum volume is 250 µl.

Why are there eight syringes if they are controlled together?

Using a separate syringe for each channel on the flow cell makes it easier to evenly control the fluidics, resulting in flow uniformity.

There are bubbles in my syringes. Is this a problem?

Small bubbles are not a problem but large bubbles can affect the flow rate across the flow cell.

I have huge bubbles in my syringes and they will not come out with repeated washings. How do I get them out?

Introduction of 0.05% Tween-20 in deionized water can help clear bubbles from the syringes.

Instrument

How do I initialize the Genome Analyzer?

Close and reopen the software. When you start a task, the instrument will initialize automatically.

Can I change the length of the tubing in the Genome Analyzer to decrease the dead volume and save costs on reagents?

Illumina does not recommend that you do so, and cannot provide support for systems that have been modified in this way.

What is the life expectancy of a manifold? Can I order it and change it out myself?

The life expectancy is not known at this time. Changing the manifold is performed by an Illumina Field Service Engineer or Field Application Scientist.

Technology Overview and Molecular Biology

Instead of sequencing the entire genome, can you narrow down the region that you wish to sequence to a candidate gene region? Do you simply clone that region and use that as your sequencing template instead of whole genomic DNA?

For a targeted approach, you can use PCR products that are derived from your region of interest as your starting point. The PCR products can be long-range PCR products or as short as 1500bp. Illumina has performed many experiments that target specific regions, and generally cover these by pooling multiple overlapping long-range PCR products.

The sample preparation for sequencing does not require cloning. The amplified target DNA goes through a process of fragmentation, end repair, adaptor ligation, size selection, and PCR enrichment. This process takes one day and allows you to process samples in parallel.

What sequencing redundancy is recommended?

This depends on the size of the organism you are trying to resequence. For whole genome resequencing, a 25-fold oversampling should be adequate. For targeted resequencing involving mixes of many PCR products, 75-fold oversampling will correct for the inability to mix the PCR products at a 1:1 ratio.

Illumina sample prep shows no systematic bias. In sequencing the X chromosome we achieved 16-fold average coverage, with all sequenceable bases covered at least twice.

What is your base-pair read length?

Currently, kits support 17, 25, and 35 bp read lengths.

What is paired-end analysis?

Paired-end analysis involves sequencing both ends of a fragment of DNA. If the fragments are of known size, this method can facilitate de novo sequencing of repetitive elements and help to identify structural variation.

How do you ensure that different adaptors are ligated to each end of a DNA fragment? What percentage of sequences have the same adaptors?

We have a proprietary method that ensures ligation of two different adaptors in the required orientation to opposing ends of a DNA fragment. PCR selects for these and finalizes the construct ready for hybridizing onto the flow cells surface. The adaptor sequences could be determined by sequencing the ligation fragments, but sequence information alone is not sufficient to uncover the method.

How much DNA is required to load a flow cell lane for bridge PCR?

We start the sample prep if possible with 1–2 µg of DNA, although we have used as a little as 0.1 µg of genomic DNA and made successful sample preparations. 1–2 µg is enough for many, many flow cells. To each flow cell, for high density clusters, we use about 100 µl of a 3 pM solution of the prepared sample (i.e. 3 x 10-4 pmol) per channel. We do not know how much of this binds to the surface, but this amount is enough to visualize around 6 million clusters in one channel, which corresponds to 6 million molecules within the viewable area. The viewable area is significantly less than the total area.

To what level have you pooled BACs successfully?

We have run customer samples where we have pooled BACs. The most we have tried so far is 29 pooled BACs of 130 kb each. There are no inherent limits in the software that would prevent this.

Do homopolymers and repetitive DNA regions impact sequencing efficiency?

Homopolymers do not impact sequencing.

The repetitive DNA content of plants is an important element. The number of uniquely alignable reads is a function of the repeat content, so this will have an impact on productivity. With longer reads and paired end in the future this may be less of an issue.

What additional equipment is required to run the protocols (sequencing, RNA, siRNA)?

Each sample preparation guide and site preparation guide contains a list of all required equipment. We are also working on a list of common lab equipment and consumables that are assumed to be available in the lab.

Additional Applications

Will we be able distinguish splice variants?

We are currently targeting the 3' ends so this is unlikely as the splice variation may likely be 5' of the fragment we sequence. We are working on a whole transcriptome sequencing method to address splice variants.

How do you analyze gene expression tag data?

Our depth of sequencing allows digital counting of transcripts in a way similar to SAGE and MPSS. We capture a small region from every transcript in your RNA sample and count how many times we see the same one. This identifies the relative proportion of each transcript in the mixture and allows you to compare samples. The fragment we sequence is obtained by capturing the 3'-most *Dpn*II or *Nla*III site and using this as an anchor to generate a 20 or 21 bp tag. These tags are then sequenced in millions of clusters to get the expression levels of all genes in that sample.

Kits and Training

What is included in the DNA preparation kit?

The DNA sample preparation kits vary depending on application. See the following sample prep guides:

Preparing Samples Sequencing Genomic DNA, part # 11251892

Preparing Samples for Digital Gene Expression-Tag Profiling with NlaIII, part # 11251702

Preparing Samples for Digital Gene Expression-Tag Profiling with DpnII, part # 11251729

Preparing Samples for Analysis of Small RNA, part # 11251913

Instrumentation

What is the image system setup?

The Genome Analyzer uses a three-laser system. One laser is used for autofocus and two for image acquisition, using a filter wheel to allow two channels per laser. The green laser images G and T; the red laser images C and A. Image capture uses a CCD camera.

Analysis Software and Computing Requirements

What are the storage size requirements for the sequence data output from a single run?

The system requires 1 TB to process the data from a single run. Images are acquired and stored on the instrument workstation and must then be transferred to an external computer to be analyzed by the Analysis Pipeline software, which handles image processing, base calling, and sequence alignment.

The software should run on all common Unix/Linux variants. A high-end Linux box should be adequate as the analysis computer. However, our software is compatible with Sun Grid Engine and LSF, if you wish to install it on a cluster.

The main issue to be aware of is that the instrument generates ~1 Tb of data during a full 2–3 day run. However ~70% of this is TIFF images that can potentially be sent to tape after a run is finished and you are satisfied a reanalysis is not required.

How much server space is required?

You should have at least 10 TB of data storage.

Can we see sample data to install and test the Analysis Pipeline?

Yes, sample BAC data are obtainable under NDA.

How long does it take to analyze a run?

Using a cluster of seven dual core computers, analyses run for 3–4 hours.

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