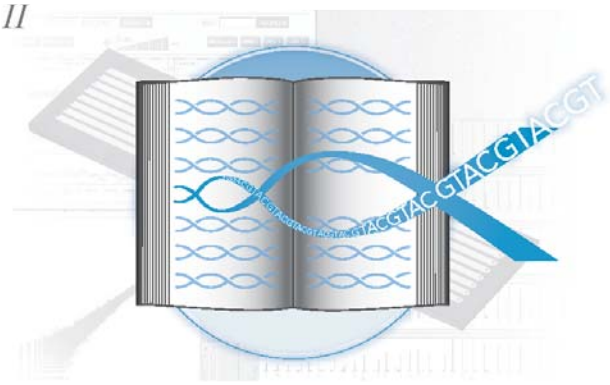
Paired-End Sequencing
User Guide

For Cluster Station and Genome Analyzer *II*

FOR RESEARCH ONLY



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Revision History

Part Number and Revision Letter	Date
1004571 Rev. A	July 2008

Table of Contents

List of Figures

List of Tables

Chapter 1
Overview

Topics

Introduction

The Illumina Genome Analysis System is a groundbreaking new platform for sequence 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, DNA-protein interaction, and small RNA identification and quantitation. Leveraging proprietary reversible terminators and Clonal Single Molecule Array technology, the Illumina Genome Analysis System can generate several 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, enables the Illumina Genome Analysis System to achieve a high degree of sequencing accuracy even through homopolymeric regions. This allows researchers to sequence complex genomes rapidly, economically, and accurately. The versatile format of the flow cell also enables researchers to tailor the system to meet the specific needs of their application.

For more information about the Illumina Genome Analysis System, refer to www.morethansequencing.com.

Audience and Purpose

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

- Operating the Illumina Cluster Station, Paired-End Module, and Genome Analyzer *II*
- 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.

Related Documentation

The following is a list of available documentation. Please check iCom (www.illumina.com/icom) or 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	Description
Sequencing Site Preparation Guide	Information about how to prepare your lab for the Cluster Station, Genome Analyzer, IPAR, and Paired-End Module. This guide includes environmental requirements, lists of user-supplied consumables, and safety hazards.
Genomic DNA Sample Prep Guide	Information about how to prepare genomic DNA samples for sequencing.
Digital Gene Expression-Tag Profiling with NlaII Sample Prep Guide	Information about how to prepare gene expression-tag samples for sequencing using *Nla*III digestion.
Digital Gene Expression-Tag Profiling with DpnII Sample Prep Guide	Information about how to prepare gene expression-tag samples for sequencing using *Dpn*II digestion.
Small RNA Analysis Sample Prep Guide	Information about how to prepare small RNA samples for analysis.
ChIP-Seq Sample Prep	Information about how to prepare genomic DNA samples for ChIP sequencing.
Single-Read Sequencing User Guide (For the Cluster Station and Genome Analyzer II)	Information about cluster generation on the Cluster Station and sequencing on the Genome Analyzer II.
Single-Read Sequencing Lab Tracking Worksheet	Printable forms where lab technicians can record lot numbers, operator names, and other information for each run.
Paired-End Sequencing User Guide (For the Cluster Station and Genome Analyzer II)	Information about paired-end reagent prep, cluster generation on the Cluster Station, and sequencing on the Genome Analyzer II.
Paired-End Sequencing Lab Tracking Worksheet	Printable forms where lab technicians can record lot numbers, operator names, and other information for each run.
Focus Procedure Experienced User Card (EUC)	Quick reference information on focusing the Genome Analyzer.
Genome Analyzer Pipeline Software User Guide	Information about how to use the Pipeline software for offline data analysis, including commands to configure the output files to meet your specific needs.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 2 *Illumina Technical Support Contacts*

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

Illumina Genome Analysis System

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

1. Sample preparation. (See *Preparing Samples for Paired-End Sequencing* on page 13.)
2. Cluster generation on the Cluster Station. (See *Using the Cluster Station* on page 31.)
3. Sequencing-by-Synthesis (SBS) on the Genome Analyzer. (See *Using the Genome Analyzer* on page 79.)
4. Data analysis using the Genome Analyzer Pipeline software. (See the *Genome Analyzer Pipeline Software User Guide* for information about data analysis.)

Sample Prep

Sequencing-by-Synthesis (SBS) can be used for multiple applications, including DNA sequencing, chromatin immunoprecipitation, whole transcriptome analysis, small RNA analysis, and digital gene expression-tag profiling. 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 an oligonucleotide coated flow cell and amplified to form a surface-bound colony (the cluster). 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 simultaneously sequence millions of clusters to generate several billion bases of data from a single run. The system leverages Illumina sequencing technology and novel reversible terminator chemistry, optimized to achieve unprecedented levels of accuracy, cost effectiveness, and throughput.

Paired-End Module

The Paired-End Module is an auxiliary instrument used to supply Read 2 reagents to the Genome Analyzer via an external VICI valve.

Flow Cell

The flow cell is a multi-lane glass-based substrate (for some flow cell types also silicon) in which clusters are generated and the sequencing reaction is performed. Each of the lanes is individually addressable, so researchers can interrogate multiple distinct samples per flow cell.

Within each lane of the flow cell, millions of primers act as capture probes for the fragmented DNA or cDNA. Each lane of the flow cell is capable of yielding millions of distinct clusters and generating several hundred Mbs 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 analyses.

There are two different types of flow cells (Figure 1):

- The entirely clear 1.4 mm flow cell with 1.4 mm wide lanes, some of which are curved at the ends. This flow cell is designed for use with the Genome Analyzer *II*, and is not compatible with older versions of the Genome Analyzer that have not been upgraded.
- The mostly black 1.0 mm flow cell with 1.0 mm wide lanes, all of which are straight. This flow cell is for use with older versions of the Genome Analyzer that have not been upgraded.

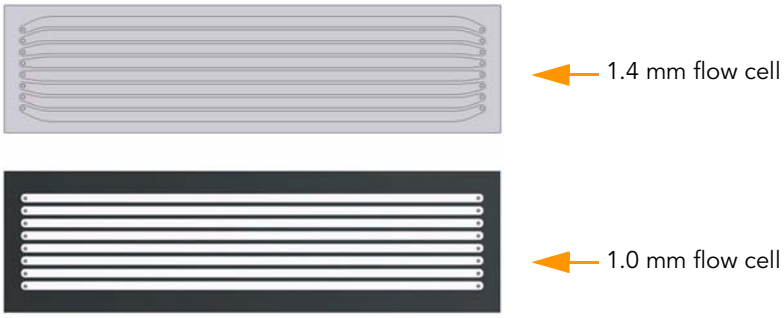


Figure 1 1.4 mm Flow Cell and 1.0 mm Flow Cell.

CAUTION

The Genome Analyzer *II* is set up to run 1.4 mm flow cells. Although it is possible to run 1.0 mm flow cells, a configuration change to the instrument that can only be performed by a Field Service Engineer is required. For contact information, see *Technical Assistance* on page 4.

The following is a list of Cluster Generation Kits containing the 1.4 mm flow cell. Please check iCom (www.illumina.com/icom) or consult with Illumina Technical Support to find out about recent updates and releases of new 1.4 mm flow cell Cluster Generation Kits.

Table 3 Paired-End Cluster Generation Kits with 1.4 mm Flow Cell

Catalog Number	Product Description
PE-203-1001	1 Paired-End Cluster Generation Kit - GA *II*
PE-203-1002	5 Paired-End Cluster Generation Kits - GA *II*

Paired-End Sequencing

This guide includes a set of protocols for the paired-end application and instructions for operating the Paired-End Module. The paired-end protocols include sample preparation, cluster amplification, Read 1 preparation, Read 2 preparation, and two rounds of SBS sequencing. Described are the steps required to enable paired-end sequencing of clusters using the Illumina paired-end method.

Check to ensure that you have the following kits and components for paired-end reads.

- Paired-End Flow Cell
- Paired-End Sample Preparation Kit
- Paired-End Cluster Generation Kit
- Paired-End Module and Software Package
- Two 36-Cycle SBS Sequencing Kits

Key Differences

The majority of the steps in this set of protocols are identical to those used in the conventional cluster sequencing, but with some key differences, enabling you to sequence both DNA strands within each cluster.

- **New software**—The Paired-End Module requires software version SCS 2.0 or later.
- **New functionalized flow cell**—In order to perform paired-end sequencing, a modified, paired-end enabled flow cell is required. Using a standard flow cell will result in an inability to perform both reads of the paired-end experiment.
- **Modified sample preparation**—Template preparation includes a new adaptor oligo mix (PE adaptor oligo mix).
- **Two new linearization methods**—Clusters are prepared for sequencing twice, once before each of the two SBS reads. The two linearization methods are different to allow selective linearization of the desired strand.
- **Different sequencing primers**—There are two hybridization events that use a different sequencing primer for each read.
- **Combined blocking steps**—Clusters prepared using the Illumina paired-end method require an additional blocking step to improve sequencing performance. To simplify the protocol, the two blocking steps have been combined into one.
- **Modified Sample Preparation Kit**—Sample preparation for paired-end libraries adds a second, unique site complementary to the new sequencing primer. The modified kit is supplied in two boxes. See *Sample Preparation Kit Contents* on page 16.
- **Paired-End Cluster Generation Kit**—This kit contains the reagents required to generate clusters on a paired-end flow cell and to prepare the clusters for Read 1 and Read 2. Reagents that are required but are not included in the kit are listed in the related section of the protocol. The kit is supplied in four boxes. See *Cluster Generation Kit Contents* on page 46.

- **Paired-End Module**—This module is an external valve attachment to the Genome Analyzer. It supplies additional reagents to the flow cell during Read 2 preparation.
- **Two 36-cycle SBS Sequencing Kits**—Each paired-end run requires two rounds of standard SBS sequencing: Read 1 and Read 2. Each read uses one standard SBS sequencing kit, supplied in two boxes and one bag. *Prepare Reagents for Read 1 on the Genome Analyzer* on page 101.

Protocol Workflow

The paired-end process sequences the same population of clusters on the same flow cell twice, as described in the following workflow:

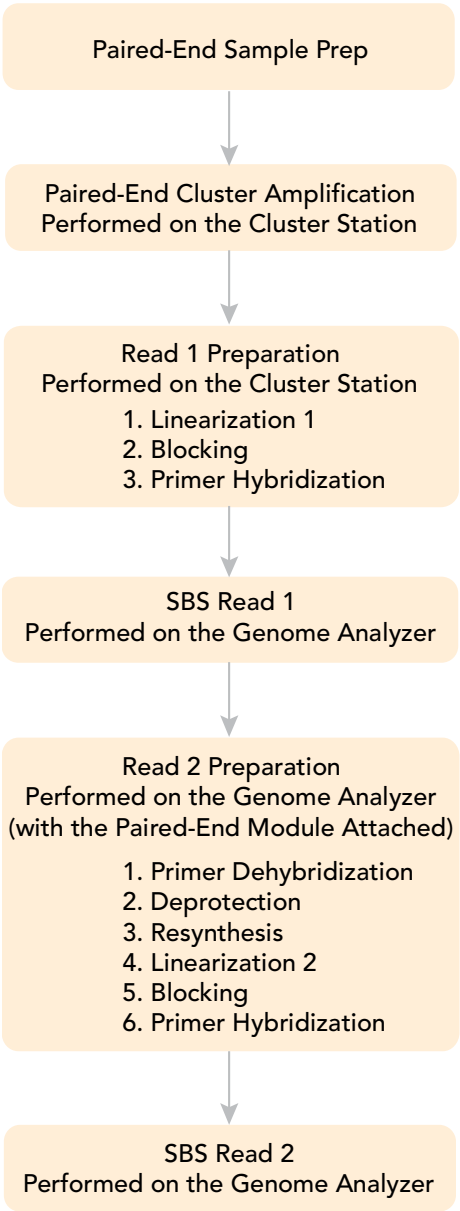


Figure 2 Paired-End Protocol Workflow

1. **Sample Preparation**—This step is identical to conventional sample preparation and cluster creation, but with a modified template. Two unique priming sites are introduced into the template during sample preparation to allow the hybridization of two sequencing primers, one in each of the two paired-end SBS reads.
2. **Cluster Amplification**—The prepared sample is introduced into the flow cell mounted on the Cluster Station, and then amplified.

   With the Illumina control PhiX library (46% GC content, average insert length 200 bp), cluster amplification should be carried out using 35 cycles of amplification. With other libraries, the density of clusters and number of amplification cycles to use should be chosen based on:

   - The GC content of the DNA sample from which the library is prepared
   - The average insert length

   As a general rule, GC-rich genomes require a higher number of amplification cycles to achieve adequate cluster intensity. Since there is a direct correlation between insert size and cluster size, libraries with longer insert sizes require a reduced density of clusters to avoid excessive overlapping of clusters.
3. **Preparation for Read 1**—The amplified sample, still mounted on the Cluster Station, is prepared for Read 1. Preparation for Read 1 is performed on the Cluster Station.
   - **Linearization 1**—Selectively linearizes one of the two strands.
   - **Blocking**—Prevents non-specific sites from being sequenced.
   - **Denaturation and hybridization**—Standard denaturation and hybridization of the first sequencing primer (Read 1 PE Sequencing Primer).
4. **Read 1 Sequencing**—The flow cell is mounted on the Genome Analyzer and subjected to 36 cycles of Sequencing-By-Synthesis, using slightly modified sequencing protocols and standard SBS reagents.
5. **Preparation for Read 2**—The flow cell is prepared for Read 2 while still mounted on the Genome Analyzer with the Paired-End Module attached, allowing for the *in situ* treatment of the flow cell.
   - **Primer Dehybridization**—Removes the extended sequencing primer used in Read 1.
   - **Deprotection**—Prepares the flow cell for the next step.
   - **Resynthesis**—Regenerates the previously linearized strand.
   - **Linearization 2**—Linearizes the strand that was sequenced in Read 1 to allow hybridization of the second sequencing primer to the newly synthesized DNA strand.
   - **Blocking**—Prevents non-specific sites from being sequenced.
   - **Denaturation and hybridization**—Denatures the linearized strand and hybridizes the second sequencing primer (Read 2 PE Sequencing Primer).
6. **Paired-End Module Wash**—This washing step is part of the Paired-End Module maintenance.

7. **Read 2 Sequencing**—The flow cell is subjected to an additional 36 cycles of SBS, using slightly modified sequencing protocols and standard SBS reagents.
8. **Post Paired-End Run Wash**—This washing step is part of the Paired-End Module and Genome Analyzer maintenance.

User Supplied Consumables and Equipment

Consumables

Check to ensure that you have all of the following user-supplied consumables.

Sample Prep

- Purified DNA (1–5 µg, 5 µg recommended)
  DNA should be as intact as possible, with an OD260/280 ratio of 1.8–2.0
- Compressed air of at least 32 psi
- Clamp (1 per nebulizer)
- PVC tubing
  - Fisher Scientific, catalog # 14-176-102
  - Nalgene Labware, catalog # 8007-0060
- Certified low range ultra agarose (BIO-RAD, part # 161-3106)
- 50X TAE buffer
- Ethidium bromide
- Loading buffer
- Low molecular weight DNA ladder (NEB, part # N3233L)
- Distilled water
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)
- MinElute PCR purification kit (QIAGEN, part # 28004)
- Disposable scalpels

Cluster Generation

- 5 M Betaine Solution
- 0.2 µm cellulose acetate syringe filter
- 30 ml syringe
- EB (10 mM Tris-Cl pH 8.5)

Betaine may be prepared in advance and stored at 4°C.

Sequencing

- Immersion oil, refractive index 1.473 (Cargille, catalog # 19570)
- Ethanol absolute
- De-ionized water (18 MOhm grade)
- 250 ml MilliQ water (for washing the Paired-End Module)

Equipment

Check to ensure that you have all of the following user-supplied equipment before proceeding to sample preparation.

- Benchtop microcentrifuge
- Benchtop centrifuge with swing-out rotor
- Dark Reader transilluminator (Clare Chemical Research, part # D195M) or a UV transilluminator
- Electrophoresis unit
- Gel trays and tank
- Thermal cycler
- 50 ml polypropylene conical tubes
- 15 ml polypropylene conical Falcon tubes
- 1.5 ml polypropylene tubes
- 1.5 ml screw-cap tubes
- 2.0 ml polypropylene tubes
- 2.0 ml screw-cap tubes
- 125 ml Nalgene bottles (4)
  (ThermoFisher Scientific, catalog # 2019-0125)

Chapter 2

Preparing Samples for Paired-End Sequencing

Topics

Introduction

This protocol explains how to prepare libraries of genomic DNA for paired-end analysis on the Illumina Cluster Station and Genome Analyzer. You will add adaptor sequences onto the ends of DNA fragments to generate the following template format:

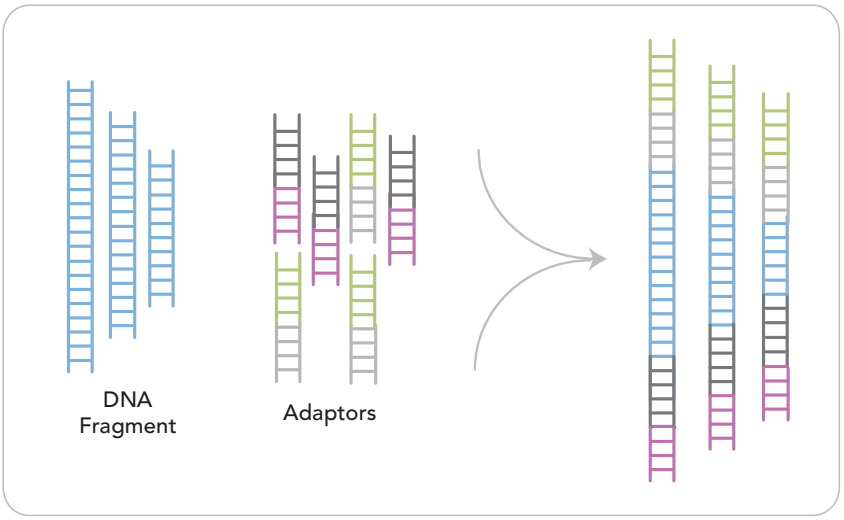


Figure 3 Fragments after Sample Preparation

The adaptors contain sequences that correspond to the two surface-bound amplification primers on the flow cells used in the Cluster Station.

Sample Prep Workflow

The following figure illustrates the steps required to prepare samples for paired-end sequencing.

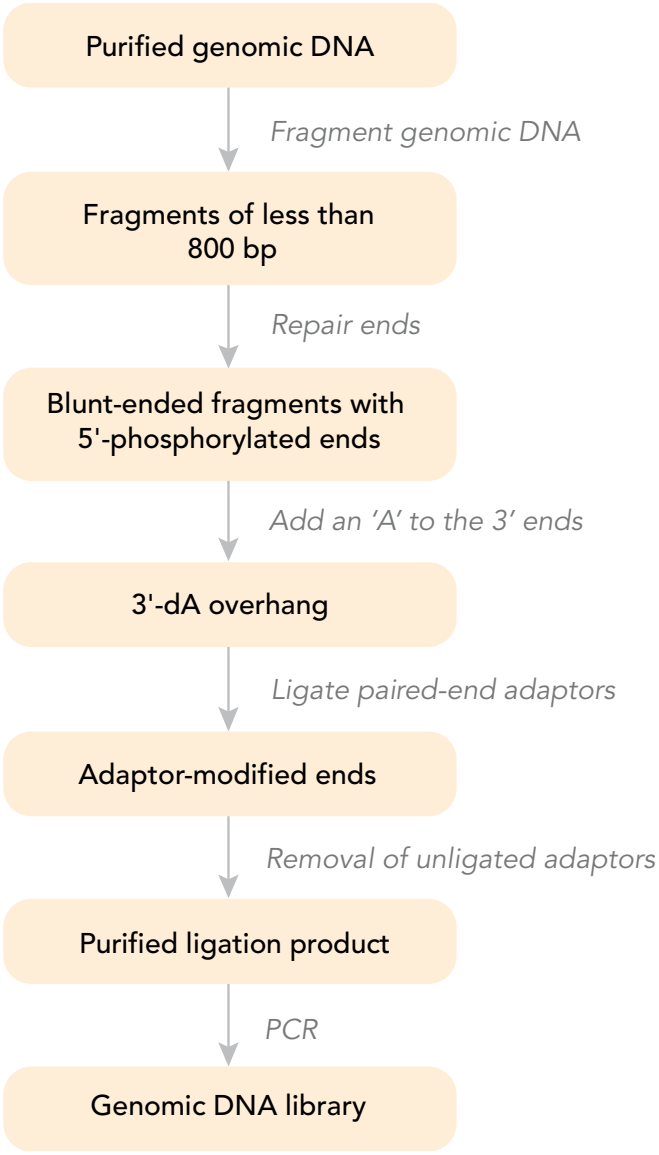


Figure 4 Sample Preparation Workflow

Sample Preparation Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

Paired-End Sample Prep Kit, Box 1

Store at -15° to 25°C

This box is shipped at -80°C. As soon as you receive it, store the components at -20°C (-15°C to -25°C).

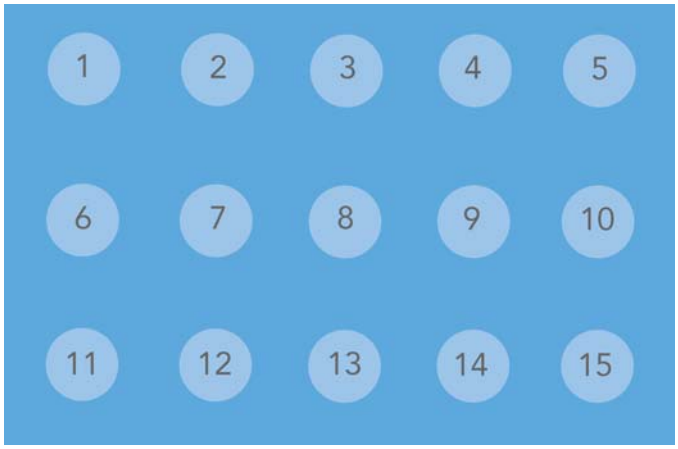


Figure 5 Paired-End Sample Prep Kit, Box 1

1. T4 DNA Ligase Buffer with 10 mM ATP, part # 1000534
2. Klenow Enzyme, part # 1000515
3. Klenow Buffer, part # 1000535
4. DNA Ligase Buffer 2X, part # 1000523
5. Phusion DNA Polymerase (Finnzymes Oy), part # 1000524
6. 10 mM dNTP Mix, part # 1001932
7. T4 PNK, part # 1000519
8. 1 mM dATP, part # 1000520
9. PE Adaptor Oligo Mix, part # 1001782
10. PCR Primer PE 1.0, part # 1001783
11. T4 DNA Polymerase, part # 1000514
12. Empty
13. Klenow Fragment (3' to 5' exo minus), part # 1000536
14. DNA Ligase, part # 1000522
15. PCR Primer PE 2.0, part # 1001784

Paired-End Sample Prep Kit, Box 2

Store at Room Temperature

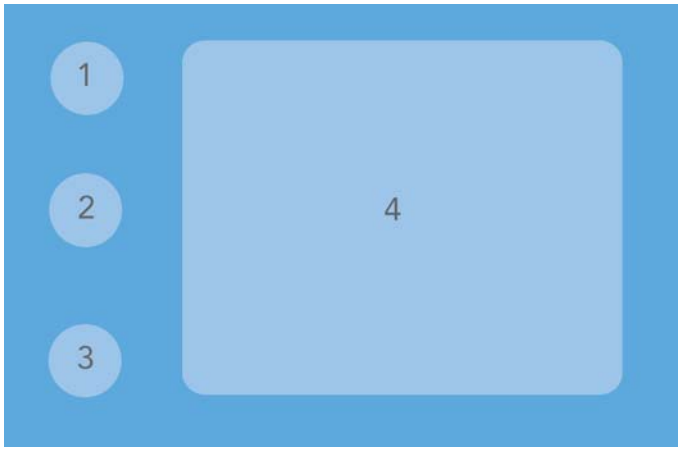


Figure 6 Paired-End Sample Prep Kit, Box 2

1. Nebulization Buffer, part # 1000466
2. TE Buffer, part # 1000465
3. Ultra Pure Water, part # 1000467
4. Nebulizer Kit (10 each), part # 1000541

Fragment Genomic DNA

This protocol fragments genomic DNA using a nebulization technique, which fragments DNA to less than 800 bp in minutes using a disposable device. Nebulization generates double-stranded DNA fragments containing of 3' or 5' overhangs.

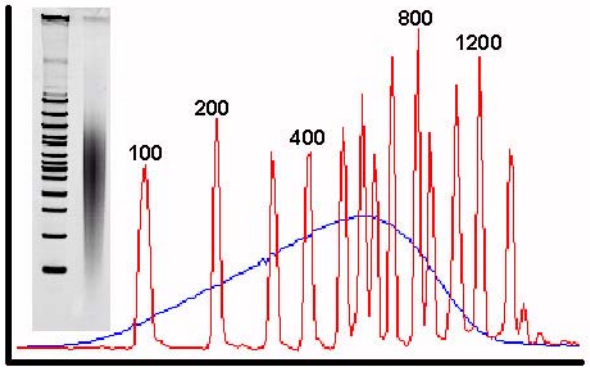


Figure 7 Fragment Genomic DNA

Consumables

Illumina-Supplied

- Nebulizers (box of 10 nebulizers and vinyl accessory tubes)
- Nebulization buffer (7 ml)
- TE Buffer

User-Supplied

- QIAquick PCR Purification Kit
- Purified DNA (1–5 µg, 5 µg recommended)
  DNA should be as intact as possible, with an OD260/280 ratio of 1.8–2.0
- Compressed air of at least 32 psi
- Clamp (1 per nebulizer)
- PVC tubing
  - Fisher Scientific, catalog # 14-176-102
  - Nalgene Labware, catalog # 8007-0060

Table 4 PVC Tubing Dimensions

ID	OD	Wall	Length
1/4 in.	3/8 in.	1/16 in.	1 meter

CAUTION

If you intend to nebulize DNA that could possibly contain any pathogenic sequences such as pathogenic viral DNA, perform the nebulization process under containment conditions (e.g., a biosafety cabinet) to prevent exposure to aerosols.

Procedure

The DNA sample to be processed should be highly pure, having an OD260/280 ratio of between 1.8 and 2.0, and should be as intact as possible.

NOTE

If you are not familiar with this shearing method, Illumina recommends that you test this procedure on test samples before proceeding with your sample DNA.

1. Remove a nebulizer from the plastic packaging and unscrew the blue lid.

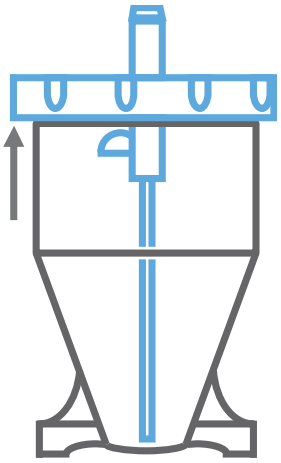


Figure 8 Remove the Nebulizer Lid

2. Using gloves, remove a piece of vinyl tubing from the packaging and slip it over the central atomizer tube. Push it all the way to the inner surface of the blue lid.

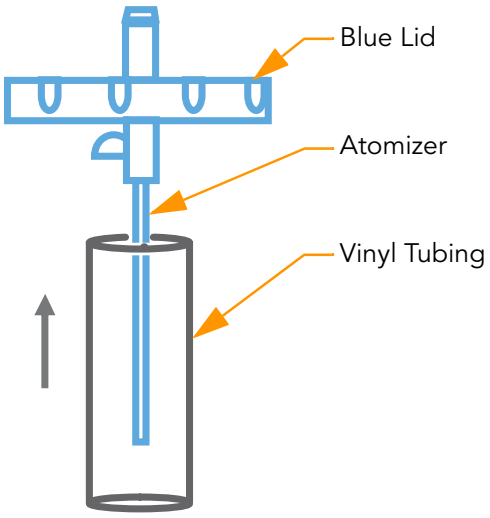


Figure 9 Assemble the Nebulizer

3. Add 1–5 µg of purified DNA in a total volume of 50 µl of TE buffer to the nebulizer.
4. Add 700 µl nebulization buffer to the DNA and mix well.
5. Screw the lid back on (finger-tight).

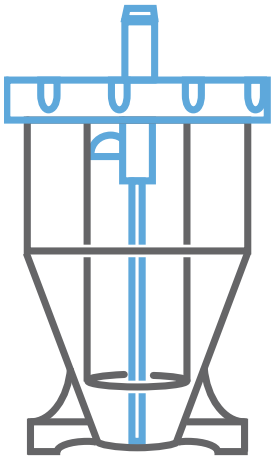


Figure 10 Replace the Nebulizer Lid

6. Chill the nebulizer containing the DNA solution on ice.
7. Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit. Secure with the small clamp.

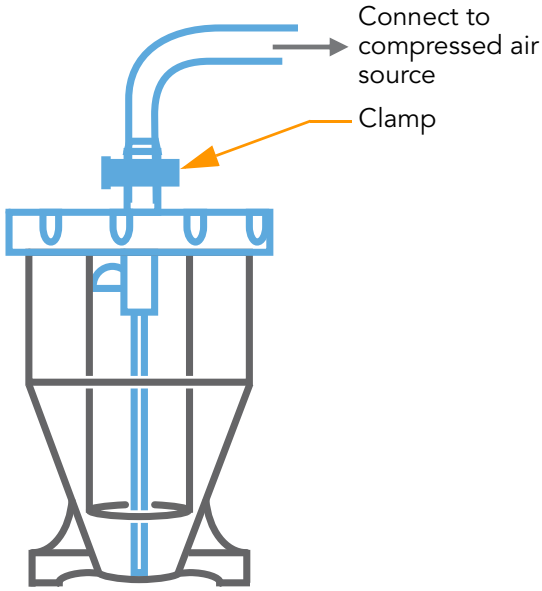


Figure 11 Connect Compressed Air

8. Bury the nebulizer in an ice bucket and place it in a fume hood.
9. Use the regulator on the compressed air source to ensure the air is delivered at 32–35 psi.
10. Nebulize for 6 minutes. You may notice vapor rising from the nebulizer; this is normal.
11. Centrifuge the nebulizer at 450 xg for 2 minutes to collect the droplets from the side of the nebulizer. If necessary, use an old nebulizer as a counter-balance.

12. If a centrifuge is not available, then use 2 ml of the binding buffer (PB or PBI buffer) from the QIAquick PCR Purification Kit to rinse the sides of the nebulizer and collect the DNA solution at the base of the nebulizer.
13. Measure the recovered volume. Typically, you should recover 400–600 µl.
14. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 30 µl of EB.

Perform End Repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and Klenow enzyme. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables

Illumina-Supplied

- T4 DNA ligase buffer with 10mM ATP
- 10 mM dNTP mix
- T4 DNA polymerase
- Klenow enzyme
- T4 PNK
- Water

User-Supplied

- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

1. Prepare the following reaction mix:
   - DNA sample (30 µl)
   - Water (45 µl)
   - T4 DNA ligase buffer with 10mM ATP (10 µl)
   - 10 mM dNTP mix (4 µl)
   - T4 DNA polymerase (5 µl)
   - Klenow enzyme (1 µl)
   - T4 PNK (5 µl)

   The total volume should be 100 µl.
2. Incubate in a thermal cycler for 30 minutes at 20°C.
3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 32 µl of EB.

Add ‘A’ Bases to the 3' End of the DNA Fragments

This protocol adds an ‘A’ base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments to be ligated to the adaptors, which have a single ‘T’ base overhang at their 3' end.

Consumables

Illumina-Supplied

- Klenow buffer
- 1 mM dATP
- Klenow exo (3' to 5' exo minus)

User-Supplied

- MinElute PCR Purification Kit (QIAGEN, part # 28004)

This protocol requires a QIAquick MinElute column rather than a normal QIAquick column.

Procedure

1. Prepare the following reaction mix:
   - DNA sample (32 µl)
   - Klenow buffer (5 µl)
   - 1 mM dATP (10 µl)
   - Klenow exo (3’ to 5’ exo minus) (3 µl)

   The total volume should be 50 µl.
2. Incubate in a thermal cycler for 30 minutes at 37°C.
3. Follow the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 10 µl of EB.

Ligate Adaptors to DNA Fragments

This protocol ligates adaptors to the ends of the DNA fragments, preparing them to be hybridized to a flow cell.

Consumables

Illumina-Supplied

- DNA ligase buffer, 2X
- PE adaptor oligo mix
- DNA ligase

User-Supplied

- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

This procedure uses a 10:1 molar ratio of adaptor to genomic DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation. If you started with less than 5 µg, reduce the volume of adaptor reagent accordingly to maintain the 10:1 ratio of DNA.

1. Prepare the following reaction mix:
   - DNA sample (10 µl)
   - DNA ligase buffer, 2X (25 µl)
   - PE adaptor oligo mix (10 µl)
   - DNA ligase (5 µl)

   The total volume should be 50 µl.
2. Incubate in a thermal cycler for 15 minutes at 20°C.
3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 30 µl of EB.

Purify Ligation Products

This protocol purifies the products of the ligation reaction on a gel to remove all unligated adaptors, remove any adaptors that may have ligated to one another, and select a size-range of templates to go on the cluster generation platform.

Consumables

User-Supplied

- Certified low range ultra agarose (BIO-RAD, part # 161-3106)
- 50x TAE buffer
- Distilled water
- Ethidium bromide
- Loading buffer (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose)
- Low molecular weight DNA ladder (NEB, part # N3233L)
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination between libraries.

It is important to perform this procedure exactly as described, to ensure reproducibility.

It is important to excise as narrow a band as possible from the gel during gel purification. Paired-end libraries should consist of templates of the same size or nearly the same size, and as narrow a size range as possible.

Illumina recommends that a Dark Reader is used to visualize DNA on agarose gels.

1. Prepare a 150 ml, 2% agarose gel with distilled water and TAE. Final concentration of TAE should be 1X at 150 ml.
2. Add ethidium bromide (EtBr) after the TAE-agarose has cooled. Final concentration of EtBr should be 400 ng/ml (i.e., add 60 µg EtBr to 150 ml of 1X TAE).
3. Cast the gel in a tray that is approximately 14 cm in length. No ethidium bromide is required in the running buffer.
4. Add 3 µl of loading buffer to 8 µl of the ladder.
5. Add 10 µl of loading buffer to 30 µl of the DNA from the purified ligation reaction.
6. Load all of the ladder solution onto one lane of the gel.

7. Load the entire sample onto another lane of the gel, leaving a gap of at least one empty lane between ladder and sample.
8. Run gel at 120 V for 120 minutes.
9. View the gel on a Dark Reader transilluminator or a UV transilluminator.
10. Place a clean scalpel vertically above the sample in the gel at the desired size of the template.
11. Excise a 2 mm slice of the sample lane at approximately 300 bp using the markers as a guide.
12. Follow the instructions in the QIAquick Gel Extraction Kit to purify on one QIAquick column, eluting in 30 µl of EB.
13. Discard the scalpel.

Enrich the Adaptor-Modified DNA Fragments by PCR

This protocol uses PCR to selectively enrich those DNA fragments that have adaptor molecules on both ends, and to amplify the amount of DNA in the library. The PCR is performed with two primers that anneal to the ends of the adaptors. The number of PCR cycles is minimized to avoid skewing the representation of the library.

Consumables

Illumina-Supplied

- Phusion DNA polymerase
- PCR primer PE 1.0
- PCR primer PE 2.0
- Ultra pure water

User-Supplied

- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

This protocol assumes 5 µg of DNA input into library prep. If you use 0.5 µg, adjust the protocol as described in the following table.

Input of DNA to Library Prep	Volume of Purified Library into PCR	Volume of Water	Number of PCR Cycles
5 µg	1 µl	22 µl	10
0.5 µg	10 µl	13 µl	12

1. Prepare the following PCR reaction mix:
   - DNA (1 µl)
   - Phusion DNA polymerase (25 µl)
   - PCR primer PE 1.0 (1 µl)
   - PCR primer PE 2.0 (1 µl)
   - Ultra pure water (22 µl)

   The total volume should be 50 µl.
2. Amplify using the following PCR protocol:
   a. 30 seconds at 98°C
   b. 10 or 12 cycles of:
      10 seconds at 98°C
      30 seconds at 65°C
      30 seconds at 72°C
   c. 5 minutes at 72°C
   d. Hold at 4°C
3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 50 µl of EB.

Validate the Library

Illumina recommends performing the following quality control steps on your DNA library.

1. Determine the concentration of the library by measuring the absorbance at 260 nm. The yield from the protocol should be between 500 and 1000 ng of DNA.
2. Measure the 260/280 ratio. It should be approximately 1.8.
3. Load 10% of the volume of the library on a gel and check that the size range is as expected: a narrow smear similar in size to the DNA excised from the gel after the ligation.

   If the DNA is not a narrow smear but instead comprises a long smear of several hundred base pairs, then another gel purification step is recommended. Repeat the procedure as described in *Purify Ligation Products* on page 25.
4. To determine the molar concentration of the library, examine the gel image and estimate the median size of the library smear.
   a. Multiply this size by 650 (the molecular mass of a base pair) to get the molecular weight of fragment in the library.
   b. Use this number to calculate the molar concentration of the library.
5. Clone 4% of the volume of the library into a sequencing vector.
   a. Sequence individual clones by conventional Sanger sequencing.

The 5' ends of the library molecules are not phosphorylated and therefore require a phosphorylated vector for cloning.

   b. Verify that the insert sequences are from the genomic source DNA.

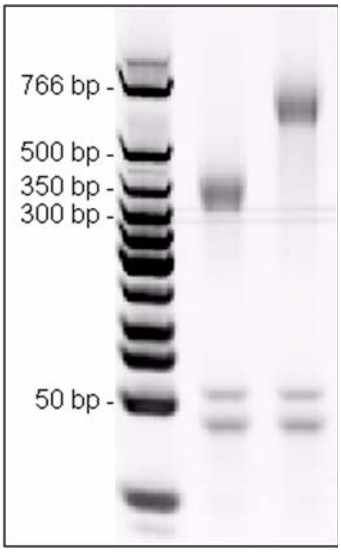


Figure 12 Library Validation Gel

This example shows a library run on a 4–20% TBE polyacrylamide gel, stained with Vistra Green (GE Healthcare # RPN5786) and visualized on a fluorescence scanner. The left lane shows a marker ladder. The center lane and right lane show paired-end libraries with insert sizes of approximately 250 bp to 550 bp, respectively. The two bands around 50 bp in size are primers from the enrichment PCR step and have no effect on the subsequent formation of clusters.

Chapter 3

Using the Cluster Station

Topics

Introduction

The Cluster Station is a fluidics 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 millions of clusters, each containing ~1,000 copies of template, in approximately 6 hours.

The Cluster Station works in conjunction with a dedicated computer and the Illumina Cluster Station software. The open-source software allows you to run individual subroutines or modify protocols to meet your research needs.

The Cluster Station automatically dispenses reagents and controls reaction times, flow rates, and temperatures.

Figure 13 Cluster Station

Cluster Generation Steps

Cluster generation consists of the following steps:

1. **Hybridize template DNA**—Hybridize template molecules onto the oligonucleotide-coated surface of the flow cell.
2. **Amplify template DNA**—Isothermally amplify the molecules to generate clonal DNA clusters.
3. **Linearize**—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.
5. **Denature and hybridize sequencing primers**—Denature the dsDNA and 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 Workflow

Table 5 *Cluster Generation Process*

Step	Instructions
1. Ensure that you have all of the required user-supplied equipment and consumables.	Refer to the booklet Cluster Station *Sequencing Site Preparation Guide*
2. Restart the Cluster Station and attached workstation.	*Starting the Cluster Station* on page 58
3. Prepare fresh reagents.	*Preparing Reagents for Cluster Generation on page 53*
4. Open and run a recipe.	*Running a Recipe* on page 59
5. Load the reagents in their appropriate positions on the Cluster Station.	*Loading Reagents for Cluster Generation* on page 56
6. Load the flow cell onto the Cluster Station.	*Positioning the Flow Cell* on page 61
7. After hybridizing the sequencing primer(s), sequence the flow cell within 4 hours.	Chapter 4, *Using the Genome Analyzer*

Protocol Times

This table shows approximately how long the Cluster Station takes to perform each step of the clustering protocol. Aside from the One Step recipe, Cluster Station recipes perform a subset of the overall procedure.

Table 6 Cluster Station Protocol Times

Step	Duration	Solution Change	Reagent (Position)	Time for Reagent
Wash	6 - 15 min *(walk-away)*			
Template Hybridization and Initial Extension	38 min *(some hands-on)*	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	2 hr 10 min (35 cycles) *(walk-away)*	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 (you can store flow cells indefinitely at 4°C)				
Linearization	58 min *(walk-away)*	9	1X Linearization Buffer* (16)	7 min
		10	Linearization 1 Mix (14)	37 min
		11	Wash Buffer (10)	7 min
		12	Storage Buffer (12)	7 min
Blocking	46 min *(walk-away)*	13	1X Blocking Buffer * (16)	7 min
		14	Blocking Mix * (8)	55 min
		15	Wash Buffer (10)	7 min
		16	Storage Buffer (12)	7 min
Denaturation and Hybridization of Sequencing Primer(s)	35 min *(walk-away)*	14	NaOH (17)	5 min
		15	TE (18)	5 min

Table 6 Cluster Station Protocol Times (Continued)

Step	Duration	Solution Change	Reagent (Position)	Time for Reagent
		16	Sequencing Primer Mix* (7)	20 min
		17	Wash Buffer (10)	5 min
			Storage Buffer (12)	
Ready for sequencing. Do not store flow cell.				
Wash	6 - 15 min *(walk-away)*			
Total Time	≈ 6h 00min	* These solutions are made fresh using kit reagents		

This flow chart shows how you can schedule the assay according to flow cell storage requirements.

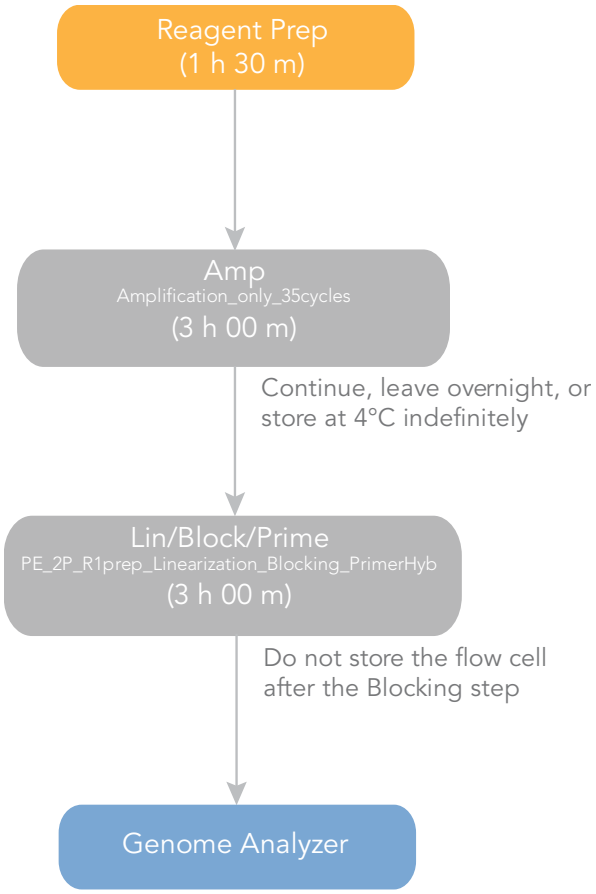


Figure 14 Scheduling the Assay

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.

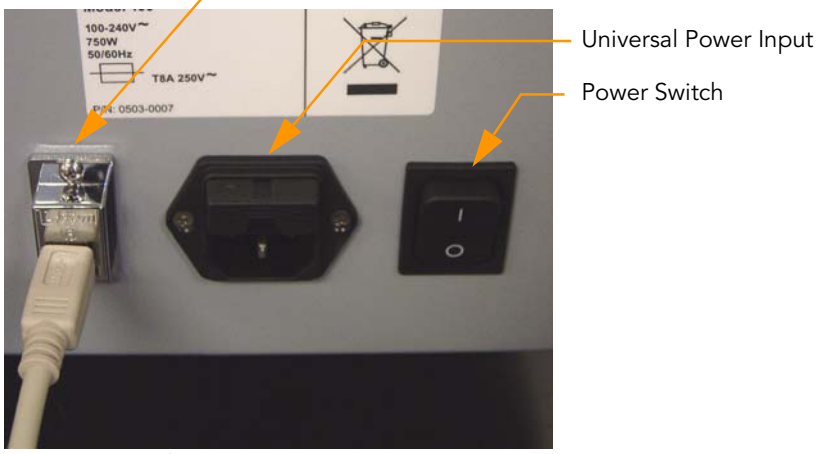


Figure 15 Cluster Station Power Connections

Instrument Areas

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

- Reagent Area
- Flow Cell Area

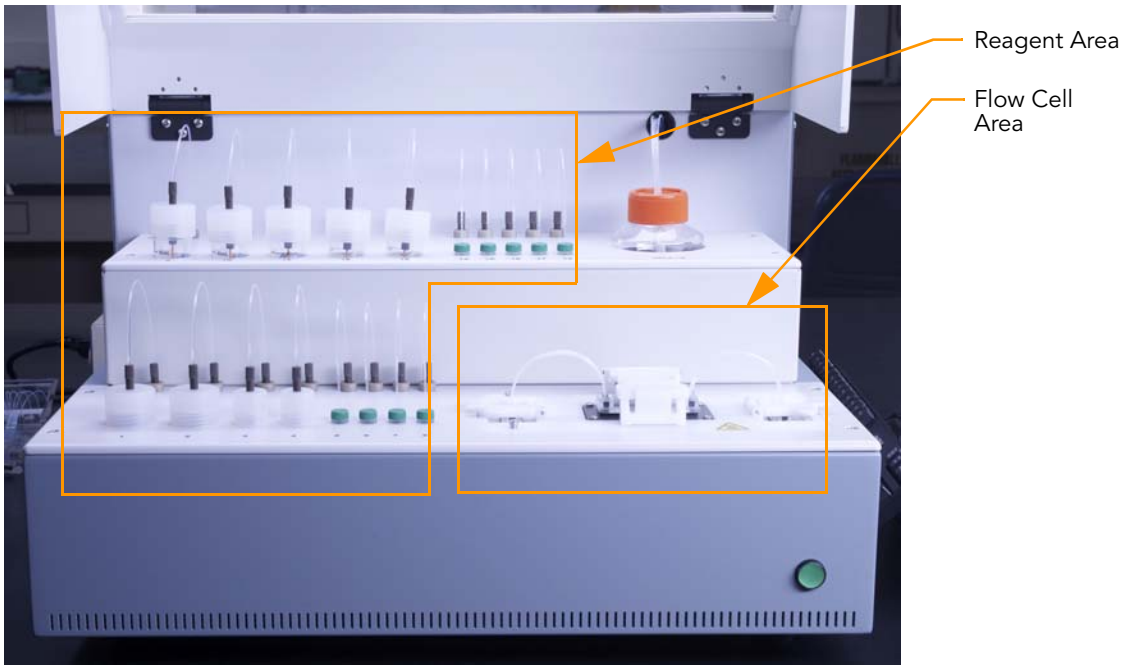


Figure 16 Cluster Station Areas

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 location.

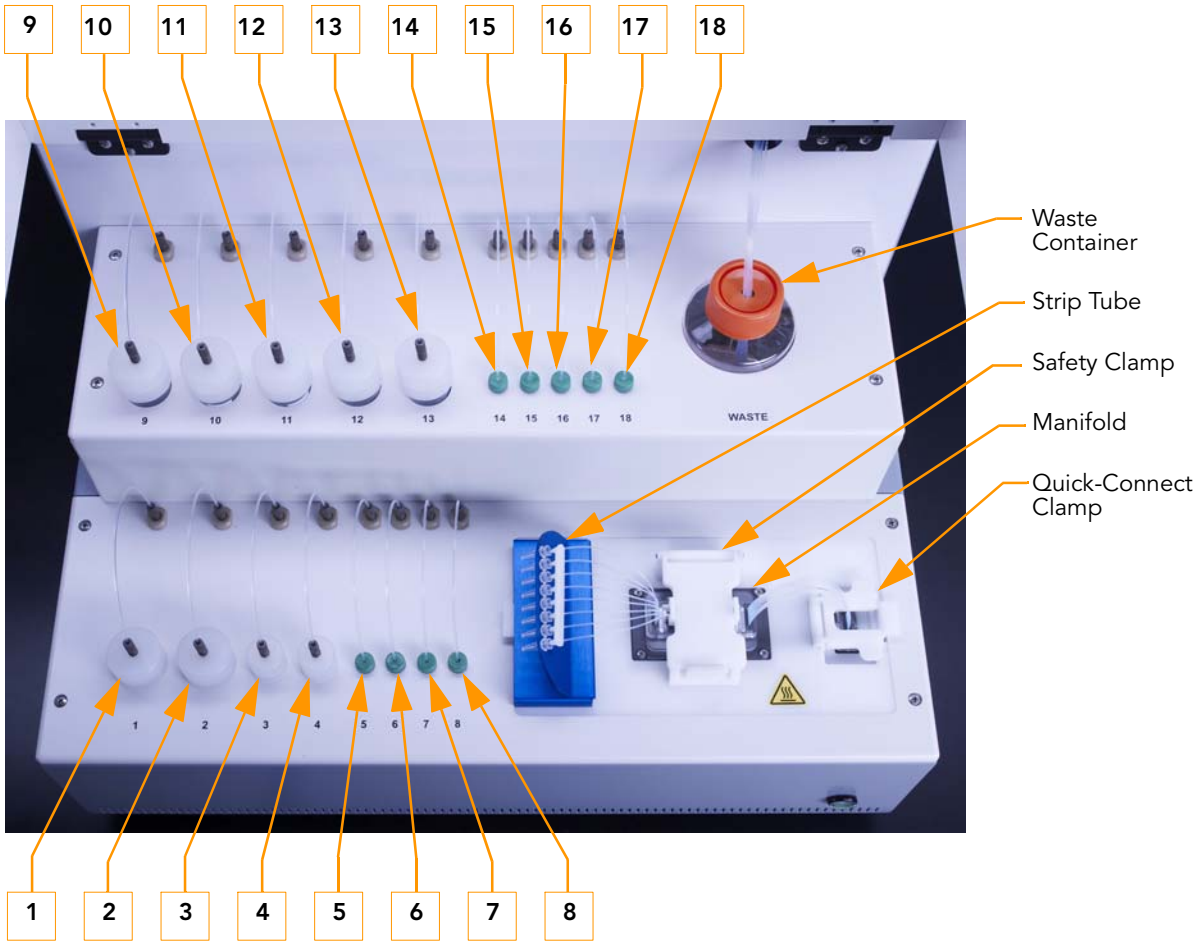


Figure 17 Positions on the Cluster Station

Waste Container

The 250 ml waste container collects the reagents after they have gone through the flow cell 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 18 Liquid Waste Container on the Cluster Station

Fluid Handling Lines

There is about 400 µl of space in the lines between the reagent bottles and the 26-way valve inside the Cluster Station, the area that needs to be primed. The volume from the 26-way valve to the flow cell is approximately 45–50 µl, while the inlet tubes on a hybridization manifold are around 35 µl per lane.

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 deliver 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.

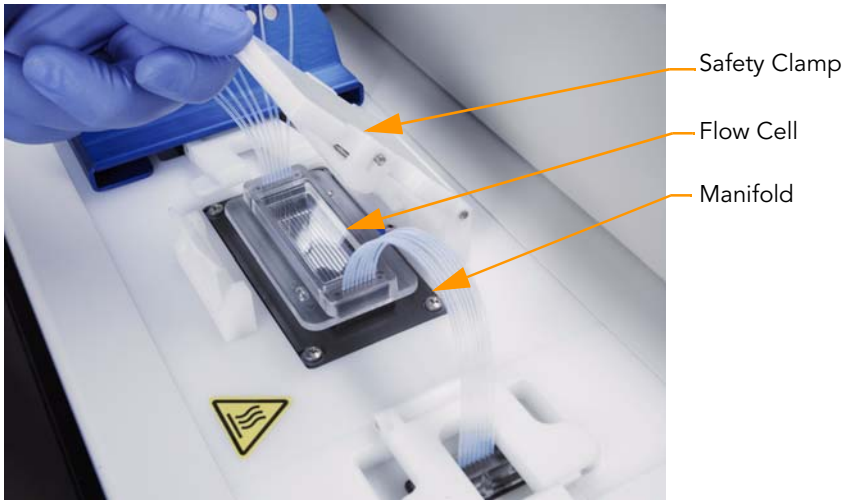


Figure 19 Flow Cell Area Components

Manifolds

Hybridization Manifold

The hybridization manifold is a disposable item used for sample loading and hybridization, and for hybridizing multiple sequencing primers. 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.

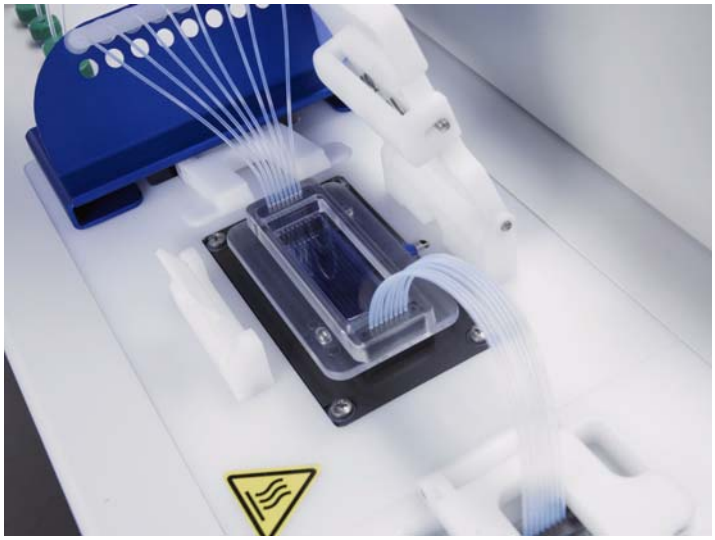


Figure 20 Flow Cell with Strip Tube and Hybridization Manifold

Amplification Manifold

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

NOTE

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.

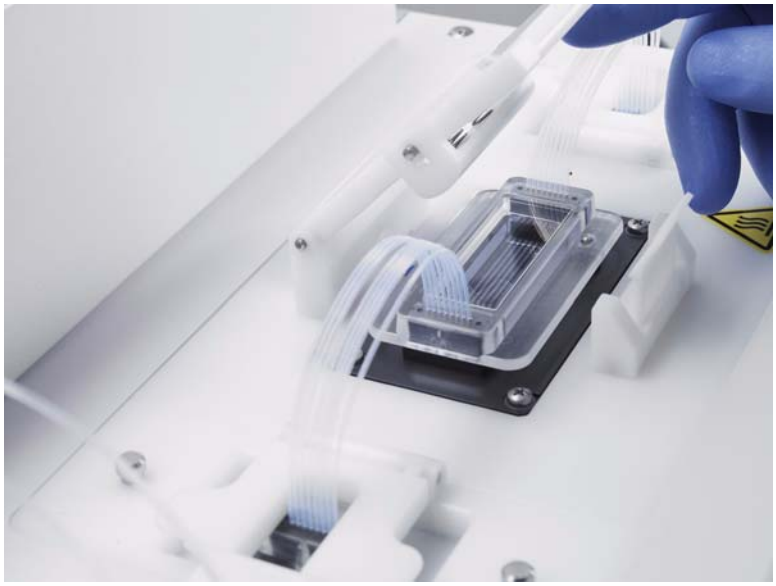


Figure 21 Flow Cell with Amplification Manifold

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.

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 amplification, hybridization and washing bridge manifolds have rubber gaskets at both ends. 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.

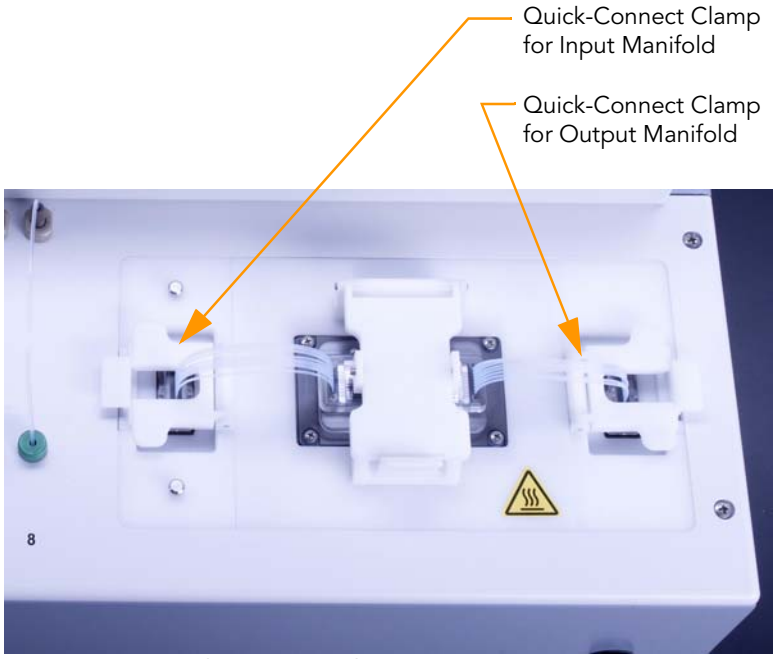


Figure 22 Quick-Connect Clamps

Washing Bridge

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

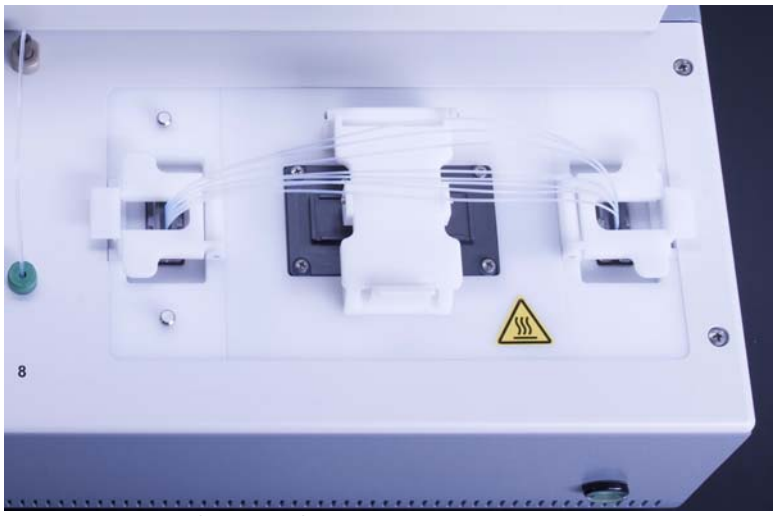


Figure 23 Washing Bridge

Cluster Station Recipes

The Cluster Station workstation is preloaded with paired-end protocol recipes. For approximate duration of each step in the protocol, see *Protocol Times* on page 35.

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_only_35cycles_v<#>
2. PE_2P_R1prep_Linearization_CombinedBlocking_PrimerHyb_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 7 Tasks in Each Cluster Station Recipe

Recipe	Wash	Hybridize and Amplify Template DNA	Linearize	Block	Denature DNA and Hybridize Sequencing Primer(s)	Wash
Amplification_only_35cycles_v<#>	X	X				X
PE_2P_R1prep_Linearization_ CombinedBlocking_Primerhyb_v<#>	X		X	X	X	X

Cluster Generation Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding to cluster generation.

Paired-End Cluster Generation Kit, Box 1 (Read 1)

Store at -15° to -25°C

This box is shipped at -80°C. As soon as you receive it, store the components at -20°C (-15° to -25°C). Briefly vortex and centrifuge each reagent after thawing.

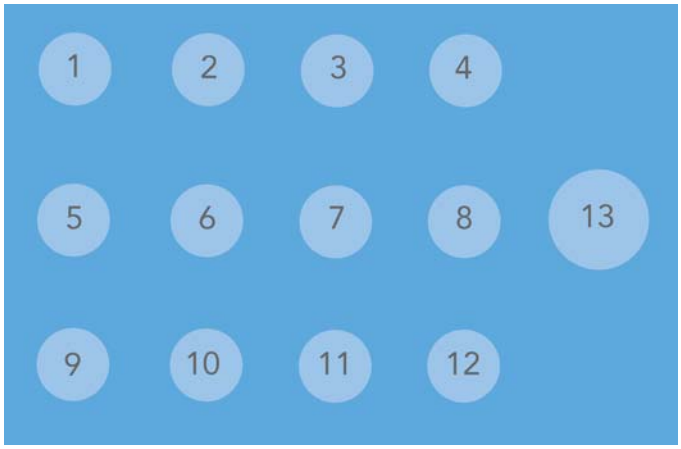


Figure 24 Paired-End Cluster Generation Kit, Box 1

1. 10 mM dNTPs, part # 1000151
2. *Bst* DNA Polymerase, part # 1000150
3. *Taq* DNA Polymerase, part # 1000157
4. 10X Blocking Buffer, part # 1001790
5. 2.5 mM ddNTP Mix, part # 1003184
6. Blocking Enzyme A, part # 1001788
7. Rd 1 PE Seq Primer, part # 1004454
8. Blocking Enzyme B, part # 1001791
9. Linearization 1 Enzyme, part # 1001796
10. 10X Linearization 1 Buffer, part # 1001797
11. Empty
12. Empty
13. Cluster Buffer, part # 1000149

Paired-End Cluster Generation Kit, Box 2 (Read 2)

Store at -15° to -25°C

This box is shipped at -80°C. As soon as you receive it, store the components at -20°C (-15° to -25°C). Briefly vortex and centrifuge each reagent after thawing.

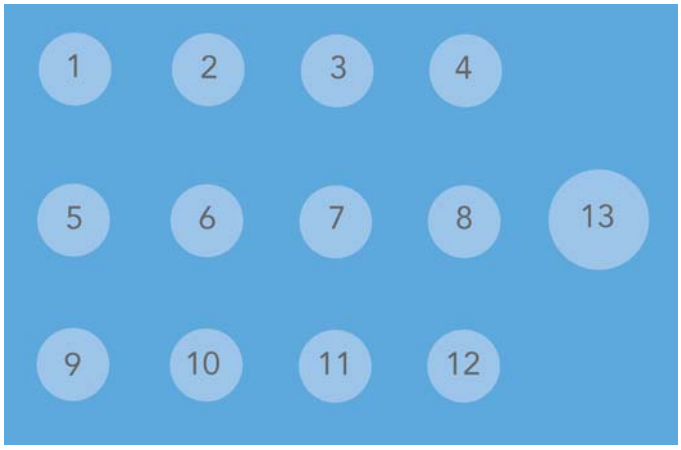


Figure 25 Paired-End Cluster Generation Kit, Box 2

1. 10 mM dNTPs, part # 1000151
2. *Bst* DNA Polymerase, part # 1000150
3. BSA, part # 1003183
4. 10X Blocking Buffer, part # 1001790
5. 2.5 mM ddNTP Mix, part # 1003184
6. Blocking Enzyme A, part # 1001788
7. Rd 2 PE Seq Primer, part # 1004055
8. Blocking Enzyme B, part # 1001791
9. Linearization 2 Enzyme, part # 1003182
10. 10X Linearization 2 Buffer, part # 1003181
11. 5X Deprotection Buffer, part # 1003179
12. Deprotection Enzyme, part # 1003180
13. Cluster Buffer, part # 1000149

Paired-End Cluster Generation Kit, Box 3 (Read 1)

Store at Room Temperature

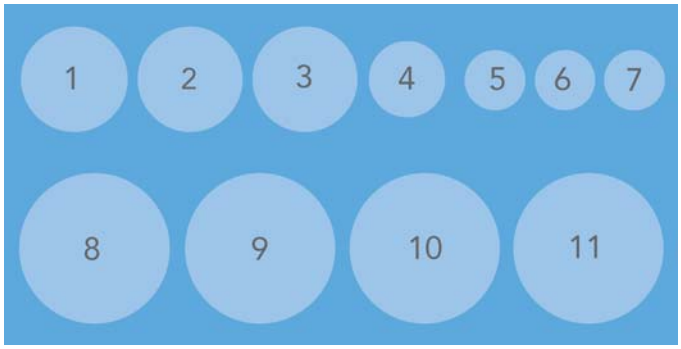


Figure 26 Paired-End Cluster Generation Kit, Box 3

1. Empty
2. Hybridization Buffer, part # 1000166
3. Empty
4. Empty
5. 2 N NaOH, part # 1000171
6. 0.1 N NaOH, part # 1000169
7. TE Buffer, part # 1000172
8. Formamide, part # 1000173
9. Wash Buffer, part # 0801-1002
10. Ultra Pure Water, part # 1000168
11. Storage Buffer, part # 1000174

Paired-End Cluster Generation Kit, Box 4 (Read 2)

Store at Room Temperature

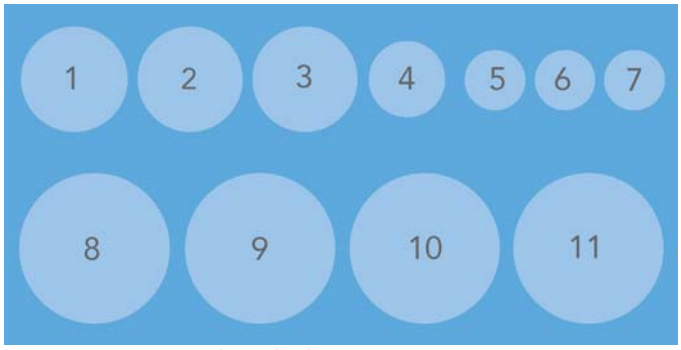


Figure 27 Paired-End Cluster Generation Kit, Box 4

1. 0.1 N NaOH, part # 1003185
2. Hybridization Buffer, part # 1000166
3. TE Buffer, part # 1003186
4. Empty

5. Empty
6. Empty
7. Empty
8. Formamide, part # 1000173
9. Wash Buffer, part # 0801-1002
10. Ultra Pure Water, part # 1000168
11. Empty

Other Cluster Station Consumables

When you order a Cluster Generation Kit, you will also receive the following consumables. The quantity varies depending on the kit size.

Illumina Part #	Description	Consumption Rate
1004225	Paired-End Flow Cell, 1.4 mm (Genome Analyzer *II*)	1 per run
0801-1320	Hybridization Manifold	1 per run
0801-1321	Amplification Manifold	2 per run

Preparing Sample DNA for Cluster Generation

There are two steps involved in preparing the template mix:

1. Denature with NaOH.
2. Dilute Denatured DNA into Hybridization Buffer.

Consumables

Illumina-Supplied

- 2 N NaOH

User-Supplied

- EB (10 mM Tris-Cl pH 8.5)

Template Mix

Prepped DNA Storage

Illumina recommends storing prepped DNA (template DNA) at a concentration of 10 nM. Adjust the concentration for your prepped DNA samples to 10 nM using EB buffer. For long-term storage of DNA samples at a concentration of 10 nM, add Tween 20 to the sample to a final concentration of 0.1% Tween. This helps to prevent adsorption of the template to plastic tubes upon repeated freeze-thaw cycles, which would decrease the cluster numbers from a sample over time.

DNA Concentration

The flow cell has eight parallel channels for processing up to eight different DNA samples. The first time you process a sample, it is useful to try a concentration range to optimize the number of clusters formed. If the DNA concentration is too low, the clusters are too few and the sequencing throughput is low. If the DNA concentration is too high, the clusters are too dense and can overlap, complicating the sequencing data analysis.

Generally, the concentration of DNA used for the hybridization step on the Cluster Station should be 1–4 pM, leading to a cluster density of approximately 40–130 K/tile.

If concentrations of DNA higher than 4 pM are required in the hybridization step, refer to *Denaturing High Concentrations of DNA* on page 51.

Denature with NaOH

Denature the template DNA with 2 N NaOH to a final DNA concentration of 0.5 nM and final NaOH concentration of 0.1 N. This is suitable for performing the hybridization step on the Cluster Station at a DNA concentration up to 4 pM.

1. If the starting DNA concentration is 10 nM, use these volumes:
   - EB (18 µl)
   - 10 nM Template DNA (1 µl)
   - 2 N NaOH (1 µl)

   The total volume should be 20 µl.

2. Vortex the template solution.
3. Pulse centrifuge the solution.
4. Incubate for 5 minutes at room temperature to denature the template into single strands.

Denaturing High Concentrations of DNA

Sporadically, higher concentrations of DNA are required in the hybridization step. In those cases, adjust the protocol *Denature with NaOH* on page 50 as indicated below.

Table 8 Adjustments to the Protocol for High Final DNA Concentrations

Desired Final DNA Concentration in 1 ml	Template DNA (10 nM)	EB	NaOH	Concentration of Denatured Template DNA
Up to 4 pM	1 µl	18 µl	1 µl	0.5 nM
4–8 pM	2 µl	17 µl	1 µl	1.0 nM
8–12 pM	3 µl	16 µl	1 µl	1.5 nM
12–16 pM	4 µl	15 µl	1 µl	2.0 nM
16–20 pM	5 µl	14 µl	1 µl	2.5 nM
20–24 pM	6 µl	13 µl	1 µl	3.0 nM
24–28 pM	7 µl	12 µl	1 µl	3.5 nM
28–32 pM	8 µl	11 µl	1 µl	4.0 nM
32–36 pM	9 µl	10 µl	1 µl	4.5 nM
36–40 pM	10 µl	9 µl	1 µl	5.0 nM

Dilute Denatured DNA with Hybridization Buffer

Dilute the denatured DNA with pre-chilled Hybridization Buffer to a total volume of 1000 µl and dispense in strip tube as described below. Illumina recommends that you perform a titration of your DNA template to determine a good density of clusters. A typical titration series would be to use a new template at 1 pM, 2 pM, and 4 pM.

Using the Genome Analyzer PhiX control, concentrations of 0.5 pM, 1 pM, and 2 pM generate cluster densities of 40K, 85K, and 130K, respectively.

1. To reach the desired final concentration for the hybridization step, dilute denatured DNA as follows:

Required Final Concentration	0.5 pM	1 pM	2 pM	4 pM
0.5 nM Denatured DNA	1 µl	2 µl	4 µl	8 µl
Pre-chilled Hybridization Buffer	999 µl	998 µl	996 µl	992 µl

CAUTION Excess NaOH in diluted samples inhibits the formation of clusters, an effect which occurs if you add more than 8 µl of the NaOH denaturation to 1 ml of hybridization buffer.

2. Vortex the template solution.
3. Pulse centrifuge the solution.
4. Add 120 µl of the Illumina control sample into tube 5 of a 0.2 ml eight-strip tube. This will place the control sample in lane 5 on the flow cell. Illumina recommends placing the control lane in this position.
5. Add 120 µl of diluted, denatured sample DNA template into the remaining tubes of a 0.2 ml eight-strip tube. Take careful note of which template goes into each tube.
6. Label the strip tube "B."
7. Set aside on ice until ready to load onto the Cluster Station.

Preparing Reagents for Cluster Generation

This protocol describes how to prepare reagents for the amplification process of cluster generation. All operations are performed on the Illumina Cluster Station.

The reagents for cluster generation are supplied in boxes 1 and 3 of the Paired-End Cluster Generation Kit. All of the reagents necessary for cluster generation on a paired-end flow cell are contained in the kit with the exception of 5 M Betaine and EB.

The reagents and materials provided are sufficient for processing one flow cell. All materials are single-use.

Follow these instructions to prepare reagents before loading them into the Cluster Station. Note that some reagents are used at more than one point during a protocol.

Avoid trapping air at the bottom of the tubes. If bubbles are present, air will be pumped instead of the reagent.

Consumables

Illumina-Supplied

The following reagents and consumables are supplied with the Paired-End Read 1 Cluster Generation Kit (Boxes 1 and 3):

- Cluster Buffer
- Formamide
- *Taq* DNA Polymerase
- *Bst* DNA Polymerase
- 10 mM dNTPs
- Hybridization Buffer
- Wash Buffer
- 2 N NaOH
- Ultra pure water
- Storage Buffer

The following consumables are also supplied:

- Hybridization manifold (1)
- Amplification manifolds (2)
- Paired-end flow cell

User-Supplied

- 5 M Betaine solution
- EB (10 mM Tris-Cl pH 8.5)

Procedure

Betaine, 5M

The addition of betaine is reported to reduce the formation of secondary structure in GC-rich regions by eliminating the base pair composition dependence of DNA melting.

Betaine may be prepared in advance and stored at 4°C.

Prepare the betaine as follows:

1. Place 400 ml water into a large beaker.
2. While mixing with a magnetic stirrer, add 585.75 g betaine in ~50 g batches.
3. Stir until the betaine has completely dissolved.
4. Incubate at 37°C for 60 minutes.
5. Adjust the volume to 1 liter with water in a volumetric flask.
6. Filter the solution with a 0.2 µm cellulose acetate filter.
7. Store at -20°C.

Hybridization Buffer

1. Aliquot 140 µl of Hybridization Buffer into each tube of an eight-strip tube.
2. Label the strip tube "A."

Wash Buffer

1. Aliquot 100 µl of Wash Buffer into each tube of an eight-strip tube.
2. Label the strip tube "C."

Amplification Premix

1. To make Amplification Premix, mix the following in a 50 ml conical tube:
   - Water (15 ml)
   - Cluster buffer (3 ml)
   - Betaine, 5M (12 ml)

   The total volume should be 30 ml.
2. Filter the Amplification Premix with a Minisart single-use 0.2 µm cellulose acetate syringe filter into a 50 ml conical tube. Label the tube "Amplification Premix."
3. Transfer 12 ml of the Amplification Premix into a 50 ml conical tube.
4. Label the conical tube "Reagent #11."
5. Add 100 µl of the Amplification Premix into each tube of a 0.2 ml eight-strip tube.

6. Label the strip tube "D."

Save the remaining Amplification Premix to prepare the initial extension mix and the amplification mix using *Bst* DNA Polymerase.

Initial Extension Mix using *Taq* Polymerase

1. To make Initial Extension Mix using *Taq* Polymerase, mix the following on ice in a 1.5 ml tube:
   - Amplification Premix (975 µl)
   - 10 mM dNTPs (20 µl)
   - *Taq* DNA Polymerase (5 µl)

   The total volume should be 1000 µl.
2. Aliquot 120 µl of Initial Extension Mix into each tube of a 0.2 ml eight-strip tube.
3. Label strip tube "E."
4. Set aside on ice until ready to load onto the Cluster Station.

Amplification Mix Using *Bst* DNA Polymerase

1. Prepare the *Bst* DNA Polymerase Amplification Mix by mixing the following in a 50 ml conical tube:
   - Amplification Premix (12 ml)
   - 10 mM dNTPs (240 µl)
   - *Bst* DNA Polymerase (120 µl)

   The total volume should be 12.36 ml.
2. Label the conical tube "Reagent #1."
3. Set aside on ice until ready to load onto the Cluster Station.

Formamide

1. Transfer 15 ml of Formamide into a 50 ml tube.
2. Label the tube "Reagent #9."

Wash Buffer

1. Transfer 10 ml of Wash Buffer into a 50 ml tube.
2. Label the tube "Reagent #10."

Storage Buffer

1. Transfer 5 ml of Storage Buffer (5 x SSC) into a 50 ml tube.
2. Label the tube "Reagent #12."

Loading Reagents for Cluster Generation

To prevent cross-contamination, follow these best practices:

- Always remove and replace 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.

The reagent compartment holds three sizes of tubes:

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

NOTE

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.

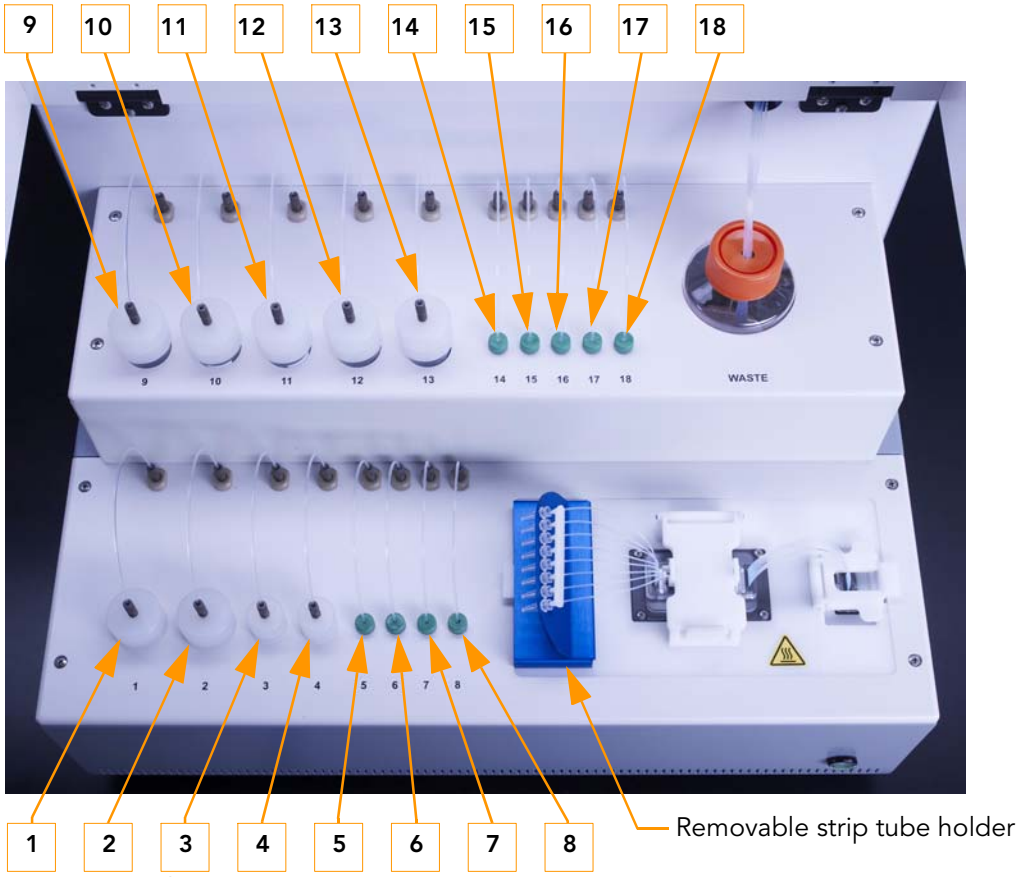


Figure 28 Cluster Station Reagent Positions

Table 9 Reagent Positions

Position	Reagent	Tube Size
1	Amplification Mix with *Bst* DNA Polymerase	50 ml
2	*Spare*	50 ml
3	*Spare*	15 ml
4	*Spare*	15 ml
5	*Spare*	1.5 ml
6	1X Blocking Buffer	2.0 ml
7	Sequencing Primer Mix	2.0 ml
8	Blocking Mix	2.0 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	Linearization 1 Mix	2.0 ml
15	*Spare*	1.5 ml
16	1X Linearization Buffer	1.5 ml
17	0.1 N 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	*Spare*	0.2 ml eight-strip tube
G	*Spare*	0.2 ml eight-strip tube
H	*Spare*	0.2 ml eight-strip tube
I	*Spare*	0.2 ml eight-strip tube
J	*Spare*	0.2 ml eight-strip tube

Starting the Cluster Station

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

CAUTION

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 using the default values:

   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.

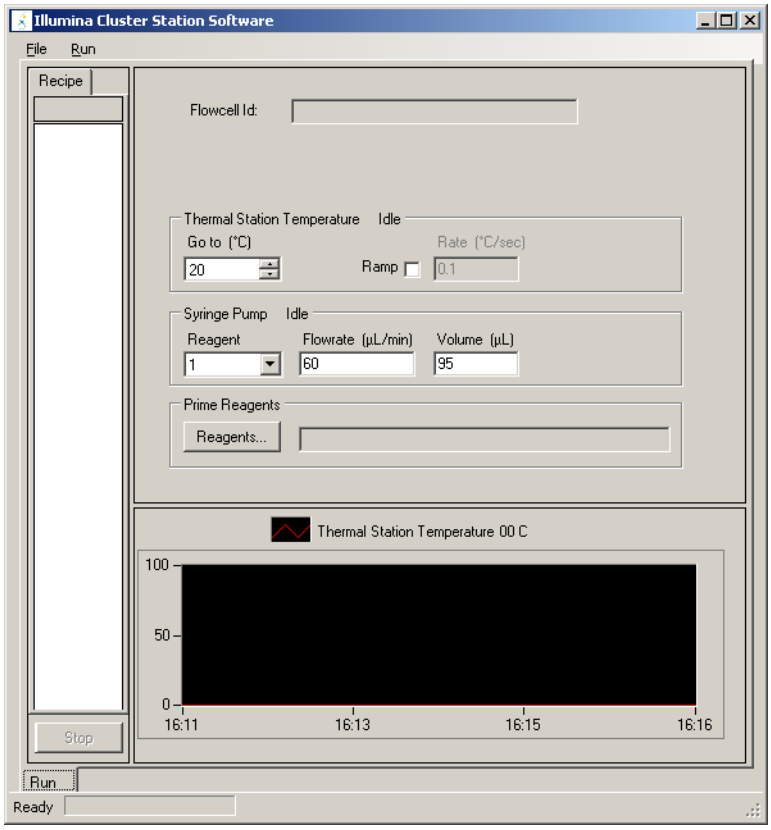


Figure 29 Cluster Station Software Main Window

Cluster Generation

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 7 on page 45.

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

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 62.

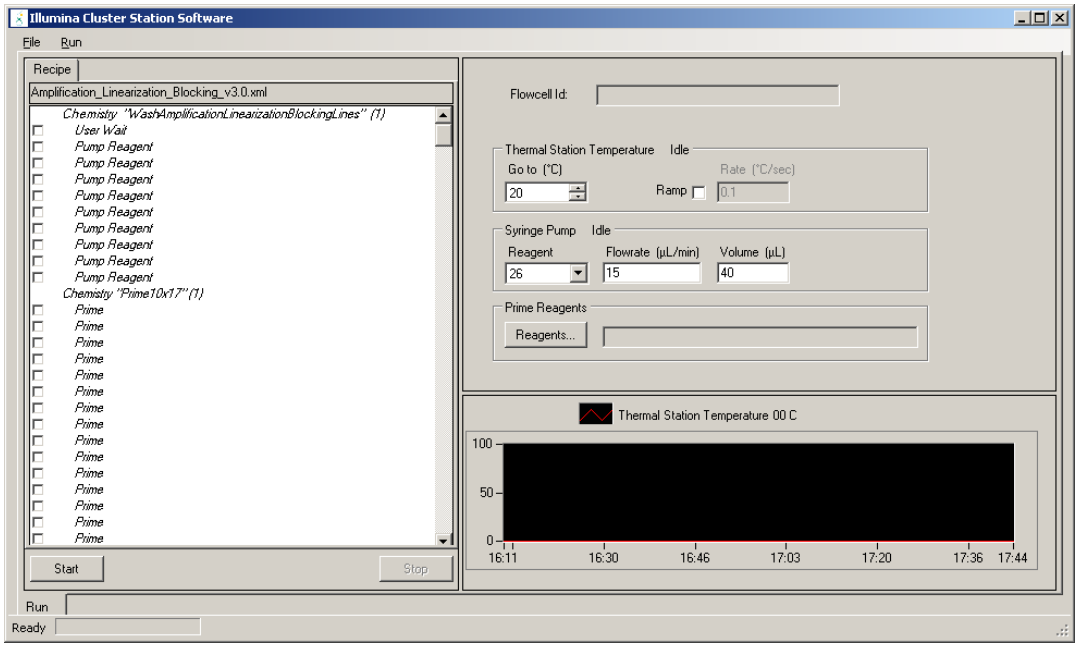


Figure 30 Amplification, Linearization, Blocking Recipe

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 202 for more information.

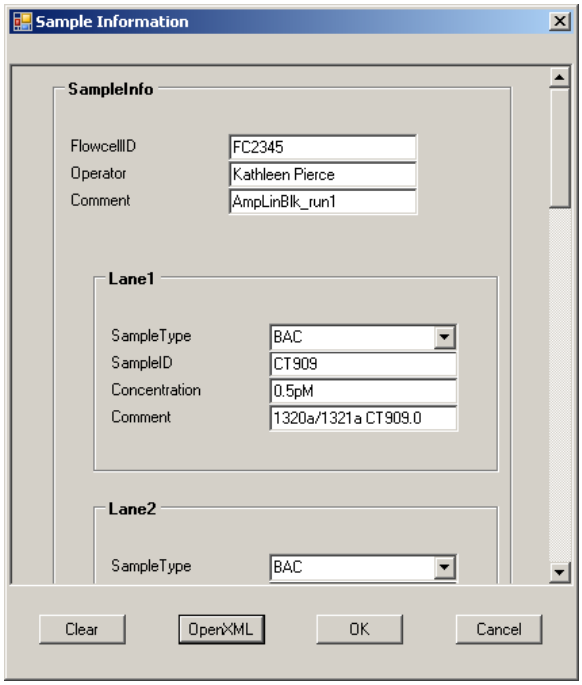


Figure 31 Sample Sheet Data

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**.

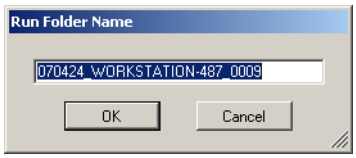


Figure 32 Run Folder

6. Follow the onscreen instructions to load reagents. Follow the guidelines in *Loading Reagents for Cluster Generation* on page 56 to position the reagents.
7. Follow the onscreen instructions to attach the hybridization manifold. For manifold descriptions, see *Manifolds* on page 40. Follow the instructions in *Attaching the Hybridization Manifold* on page 61.
8. 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. Follow the guidelines in *Check Even Flow* on page 62.
9. If the solution does not flow evenly in some or all the lines, click **Stop** to pause the protocol. Check the manifold connections and ensure that the flow cell is level. Click **Resume** to restart the protocol. If the flow continues to be uneven, try replacing the manifold.
10. When prompted, follow the onscreen instructions to remove the hybridization manifold and connect the amplification manifold. Follow the instructions in *Attaching the Amplification Manifold* on page 62.

11. When the recipe finishes, select **File | Close Recipe**.
12. 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 62 if you want to stop.

Positioning 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 the case.
2. Rinse the flow cell with water; wipe and dry the outside using lens cleaning tissue. Be careful not to drain the lanes when wiping the ports (holes). This step ensures that the flow cell does not stick to the platform.
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 bar code should be on the bottom edge, and the alphanumeric serial number on the upper edge, left side (Figure 33).

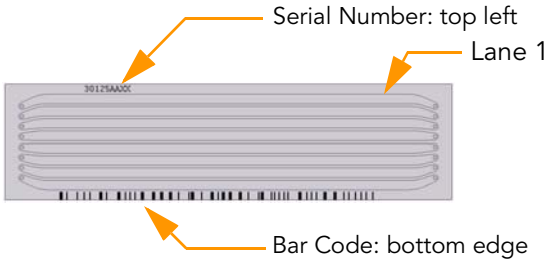


Figure 33 Positioning the Flow Cell

Attaching the Hybridization Manifold

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

1. Take a new hybridization manifold from a sealed bag. Make sure there is no dust on the underside of the gaskets (remove with a wet kimwipe or lens paper).
2. 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.
3. 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.
4. Place the fanned-out tubes into the tube strip to the left of the flow cell. You may use a piece of tape to make sure the fanned-out tubes will stay in the bottom of the tube strip.
5. Plug the clustered tubes into the output manifold to the right of the flow cell.

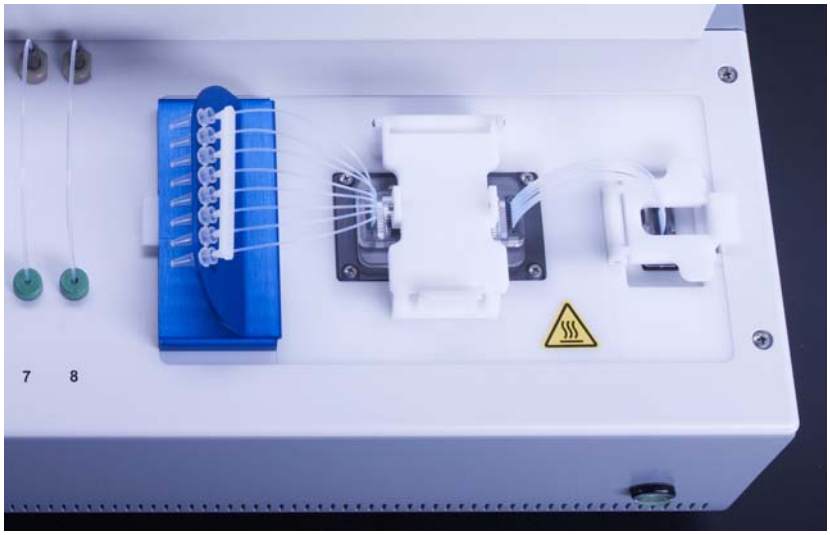


Figure 34 Flow Cell and Hybridization Manifold Installed

Check Even Flow

After you have installed the flow cell and hybridization manifold, you should check whether the flow through the flow cell is even. When the first solution is pumped through the flow cell (next section, *Running a Recipe*, step 8), make sure it runs in all eight input tubes without obstruction. The tubes should all empty at the same rate.

Attaching the Amplification Manifold

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

1. Remove the hybridization manifold.
2. Take a new amplification manifold from a sealed bag.
3. Place the center of the manifold over the flow cell.
4. 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.
5. On the amplification manifold, both ends have clustered tubes. Secure both ends (input and output) with quick-connect clamps.
6. Check that each line is priming by observing the initial air gap flowing through the lines.

Safe Stopping Points During Cluster Generation

The Cluster Station recipes give you flexibility in planning your workflow. For more information, see *Protocol Times* on page 35.

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

- Amplification_only_v<#>

For paired-reads, you can not store the linearized and blocked flow cell for a prolonged period of time due to the nature of the enzymes used in the blocking step.

Follow the instructions in *Unloading the Flow Cell* on page 63. Place the flow cell in a 50 ml conical tube filled with Storage Buffer and store it at 4°C.

NOTE

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: One of the following:

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

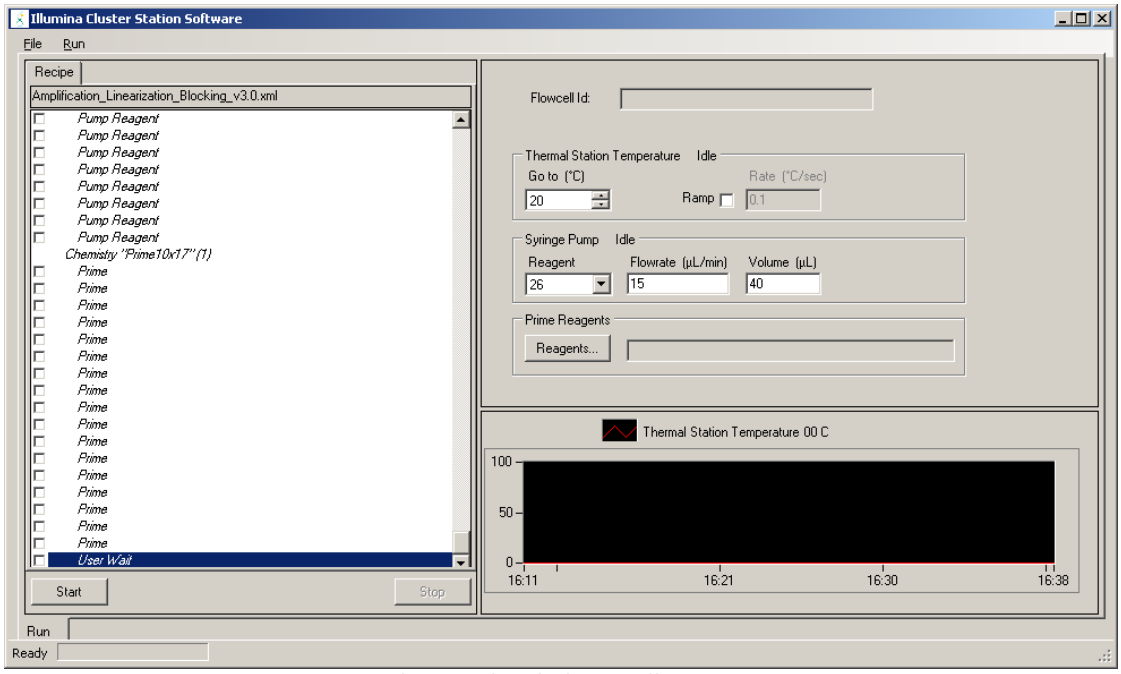


Figure 35 Setting Pump Controls to Unload Flow Cell

2. With your cursor in the **Volume** field, 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.

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_All_lines_v<#>.
3. Click **Start**.
4. Following the onscreen instructions, connect the washing bridge to the input and output manifolds.
5. Place the following tubes containing 5% DECON in the positions indicated:

Position on the Cluster Station	Tube Size	5% DECON
Positions 1, 2, 9, 10, 11, 12, and 13	50 ml	25 ml
Positions 3 and 4	15 ml	5 ml
Positions 5, 6, 7, 8, 14, 15, 16, 17, and 18	1.5 ml	1 ml

6. Click **OK**. You can reuse the 5% DECON if desired.
7. Following the onscreen instructions, replace the 5% DECON tubes with fresh tubes containing clean water in the positions indicated:

Position on the Cluster Station	Tube Size	Clean Water
Positions 1, 2, 9, 10, 11, 12, and 13	50 ml	40 ml
Positions 3 and 4	15 ml	10 ml
Positions 5, 6, 7, 8, 14, 15, 16, 17, and 18	1.5 ml	1.5 ml

8. Click **OK**.
9. Following the onscreen instructions, replace the water in each tube with the clean water in the positions indicated:

Position on the Cluster Station	Tube Size	Clean Water
Positions 1, 2, 9, 10, 11, 12, and 13	50 ml	40 ml
Positions 3 and 4	15 ml	10 ml
Positions 5, 6, 7, 8, 14, 15, 16, 17, and 18	1.5 ml	1.5 ml

10. Click **OK**.

Preparing Reagents for Read 1 Preparation on the Cluster Station

This protocol describes how to prepare reagents for the linearization, blocking, and primer hybridization process of cluster generation. All operations are performed on the Illumina Cluster Station.

Any residual Read 1 kit reagents should be disposed of after preparation of Read 1. The freezing and thawing of some components could potentially cause certain steps to fail. A separate kit for the preparation of Read 2 is supplied and contains all necessary components for the process.

Consumables

Illumina-Supplied

The following reagents and consumables are supplied with the Paired-End Read 1 Cluster Generation Kit (Boxes 1 and 3):

- 10X Linearization 1 Buffer
- Linearization 1 Enzyme
- Ultra Pure Water
- 10X Blocking Buffer
- Blocking Enzyme A
- Blocking Enzyme B
- 2.5 mM ddNTP Mix
- Hybridization Buffer
- 0.1 N NaOH
- TE Buffer
- Wash Buffer
- Storage Buffer
- Rd 1 PE Seq Primer

The following consumables are also supplied:

- Amplification manifold (1)

Procedure

Reagent #16: 1X Linearization Buffer

1. Thaw the 10X Linearization 1 Buffer at room temperature.
2. Vortex briefly.
3. Collect the reagent to the bottom of the tube by centrifuging for 10 seconds at 10,000 xg.
4. In a 15 ml conical tube, dilute the 10X Linearization 1 Buffer to a 1X concentration with Ultra Pure Water as follows:
   - Ultra Pure Water (2700 µl)
   - 10X Linearization 1 Buffer (300 µl)

   The total volume should be 3000 µl.
5. Label the tube "1X Linearization Buffer."

6. Mix by pipetting up and down using a 5 ml pipette.
7. Transfer 1.3 ml of diluted Linearization Buffer into a 1.5 ml screw-cap tube.
8. Label the tube "Reagent #16" and set aside until ready to load it onto the Cluster Station.

   Save the remaining 1X Linearization Buffer for the preparation of the Linearization Mix.

Reagent #14: Linearization 1 Mix

1. Flick the Linearization 1 Enzyme tube gently.
2. Collect the reagent to the bottom of the tube by centrifuging for 10 seconds at 10,000 xg.
3. Prepare the following reagents in a 1.5 ml screw-cap tube and mix on ice at all times:
   - 1X Linearization Buffer (1287 µl)
   - Linearization 1 Enzyme (13 µl)

   The total volume should be 1300 µl.
4. Mix by slowly pipetting up and down using a 1 ml tip.
5. Label the tube "Reagent #14" and set aside on ice until you are ready to load it onto the Cluster Station.

Reagent #6: 1X Blocking Buffer

1. Thaw the 10X Blocking Buffer at room temperature.
2. Vortex briefly.
3. Collect the reagent to the bottom of the tube by centrifuging for 10 seconds at 10,000 xg.
4. In a 15 ml conical tube, dilute the 10X Blocking Buffer to a 1X concentration with Ultra Pure Water in the following volumes:
   - Ultra Pure Water (4500 µl)
   - 10X Blocking Buffer (500 µl)

   The total volume should be 5000 µl.
5. Label the tube "1X Blocking Buffer."
6. Mix by pipetting up and down using a 5 ml pipette.
7. Transfer 2 ml of the 1X Blocking Buffer into a 2 ml screw-cap tube.
8. Label the tube "Reagent #6" and set aside until you are ready to load it onto the Cluster Station.

   Save the remaining 1X Blocking Buffer to prepare the Blocking Mix.

Reagent #8: Blocking Mix

1. Thaw the 2.5 mM ddNTP at room temperature.
2. Vortex briefly.
3. Collect the reagent to the bottom of the tube by centrifuging for 10 seconds at 10,000 xg.

4. Set aside on ice until you are ready to prepare the Blocking Mix.
5. Flick the Blocking Enzyme A and Blocking Enzyme B tubes gently.
6. Centrifuge the Blocking Enzyme A and Blocking Enzyme B tubes for 10 seconds at 10,000 xg.
7. Place the Blocking Enzyme A and Blocking Enzyme B tubes back on ice.
8. Prepare the following reagents in a 2 ml screw-cap tube and mix on ice at all times:
   - 1X Blocking Buffer (1529 µl)
   - 2.5 mM ddNTP (67 µl)
   - Blocking Enzyme A (20 µl)
   - Blocking Enzyme B (84 µl)

   The total volume should be 1700 µl.
9. Mix by slowly pipetting up and down using a 1 ml tip.
10. Label the tube "Reagent #8" and set aside on ice until you are ready to load it onto the Cluster Station.

Reagent #7: Sequencing Primer Mix

1. Mix the following reagents in a 2 ml screw-cap tube:
   - Hybridization Buffer (1313.4 µl)
   - Rd 1 PE Seq Primer (6.6 µl)

   The total volume should be 1320 µl.
2. Mix by pipetting up and down using a 1 ml tip.
3. Label the tube "Reagent #7" and set aside on ice until you are ready to load it onto the Cluster Station.

Reagent #17: 0.1 N NaOH

1. The tube of 0.1 N NaOH supplied in the kit is ready to use as reagent #17.
2. Label the tube "Reagent #17."

Reagent #18: TE

1. The tube of TE supplied in the kit is ready to use as reagent #18.
2. Label the tube "Reagent #18."

Reagent #10: Wash Buffer

1. Transfer 15 ml of Wash Buffer into a 50 ml tube.
2. Label the tube "Reagent #10."

Reagent #12: Storage Buffer

1. Transfer 10 ml of Storage Buffer (5 x SSC) into a 50 ml tube.
2. Label the tube "Reagent #12."

Loading Reagents for Read 1 Preparation on the Cluster Station

The following figure illustrates the reagent positions for Read 1 on the Cluster Station. Some of the reagent positions differ from the positions you would use for a standard run.

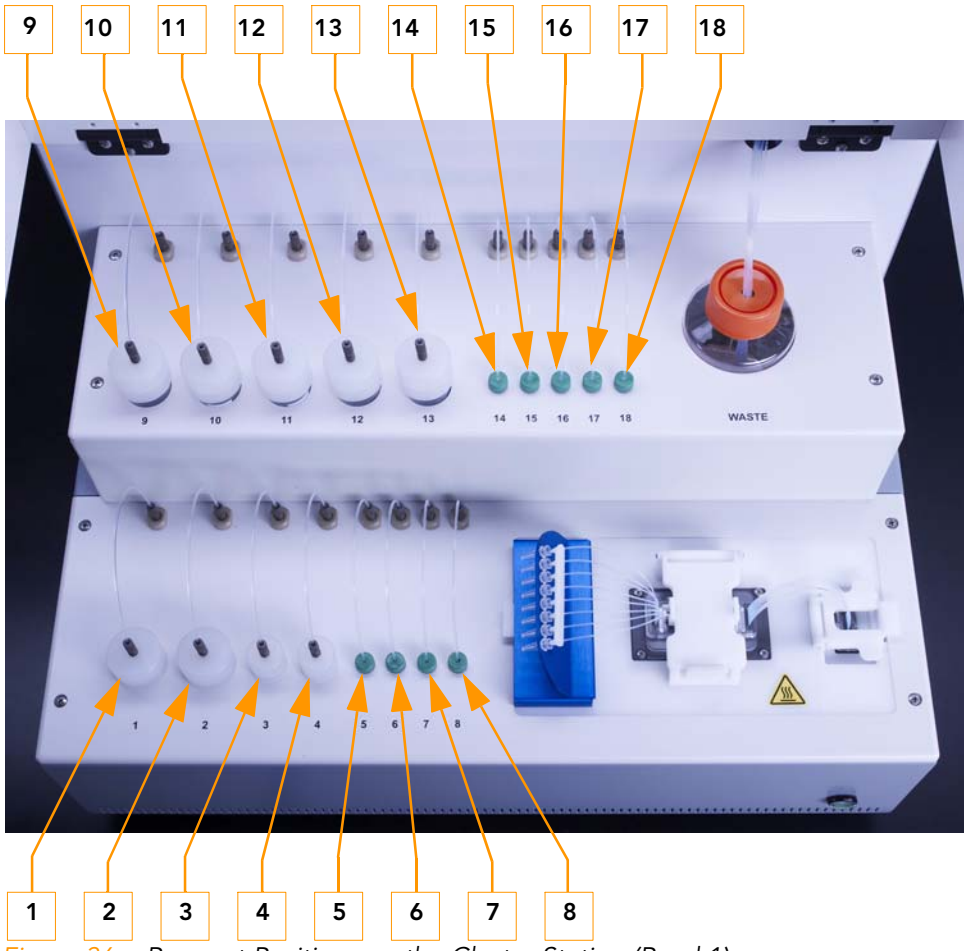


Figure 36 Reagent Positions on the Cluster Station (Read 1)

The following table lists the position each reagent occupies on the Cluster Station, the initial volume of each reagent, and the expected volume after Read 1.

Table 10 Reagent Positions on the Cluster Station and Read 1 Preparation Volumes

Position	Reagent	Initial Volume	Expected Volume After Read 1 Prep
1–5, 9, 11, 13, 15	Empty		
6	1X Blocking Buffer	2000 µl	1046 µl
7	Sequencing Primer Mix	1320 µl	326 µl
8	Blocking Mix	1700 µl	146 µl
10	Wash Buffer	15,000 µl	12,414 µl
12	Storage Buffer	10,000 µl	7414 µl
14	Linearization 1 Mix	1300 µl	234 µl
16	1X Linearization Buffer	1300 µl	234 µl
17	0.1 N NaOH	1500 µl	434 µl
18	TE	1500 µl	434 µl

Linearization, Blocking, and Primer Hybridization on the Cluster Station

The software guides you through the steps for using and loading the reagents on the Cluster Station.

Do not linearize and block a paired-end flow cell until the day of use.

Since the Blocking 2 enzyme exhibits some limited 3'–5' exonuclease activity, storing the flow cell after blocking could result in deblocking of the flow cell.

If you immediately hybridize the flow cell, the treatment of NaOH during hybridization of the sequencing primer denatures the enzyme.

1. Open the following recipe:
   PE_2P_R1prep_Linearization_CombinedBlocking_PrimerHyb_v<#>.xml.
2. Click **OK** to proceed.
3. Follow the instructions on the screen:

   "Wash lines for Read 1 linearization, blocking, and primer hybridization. Please attach washing bridge and load water in positions 6, 7, 8, 10, 12, 14, 16, 17, and 18."

   If not already in place, attach the washing bridge and load water in the positions requested.
4. Click **OK** to proceed.

   The Cluster Station washes all the lines to be used for cluster linearization, blocking, and primer hybridization.

   The following message should appear: "Washing of lines finished."
5. Click **OK** to proceed.
6. Follow the instructions on the screen:

   "Start of Read 1 linearization, blocking, and primer hybridization protocol. Please remove water from reagent positions 6, 7, 8, 10, 12, 14, 16, 17, and 18 for priming of air gap."
7. Click **OK** to proceed.

   The following message should appear:

   "Air gap primed. Load reagents in positions 6, 7, 8, 10, 12, 14, 16, 17, and 18."
8. Click **OK** to proceed.
9. Follow the instructions on the screen:

   "Please load flow cell and attach amplification manifold."

   Load the flow cell onto the thermal station and attach the amplification manifold.
10. Click **OK** to proceed.
11. As the process starts, check for correct fluid flow through all eight lines of the amplification manifold.

If the solution is not flowing in one or more lanes, readjust the amplification manifold and repeat the test for regular flow in all lanes. When the flow is regular in all lanes, proceed with the protocol.

The process can then be left to run unattended.

At the end of the process, the following message should appear:

"Flow cell is ready for sequencing on the Genome Analyzer."

12. Disconnect the manifold at the inlet, select position 26, and pump 25 µl of air to partially empty the inlet tubes.
13. Remove the flow cell from the Cluster Station.

    The flow cell is ready to be sequenced on the Genome Analyzer.
14. Wash the lines used for linearization, blocking, and primer hybridization.
    a. Fill reagent positions 6, 7, 8, 10, 12, 14, 16, 17, and 18 with water.
    b. Connect the washing manifold.

Do not store the flow cell at this point for long periods of time. It is not advisable for the flow cell to remain on the Cluster Station for more than four hours. Sequencing must be performed on the flow cell within four hours.

A weekly DECON wash is required, using the recipe DECON_Wash_All_Linesv3.0.xml.

The DECON solution consists of 5% DECON in water. All lanes are washed once with DECON solution, followed by two washes with water.

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.

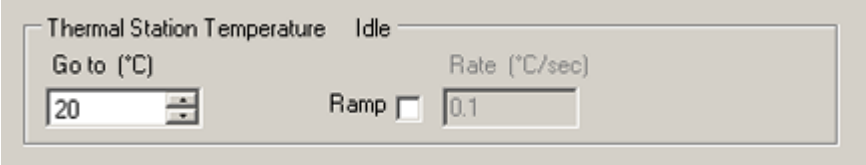


Figure 37 Thermal Station Temperature

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

NOTE

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 38 Syringe Pump

1. In the **Syringe Pump** field, select the reagent position.

   Reagents 1–18 correspond to the reagent positions illustrated in Figure 39.

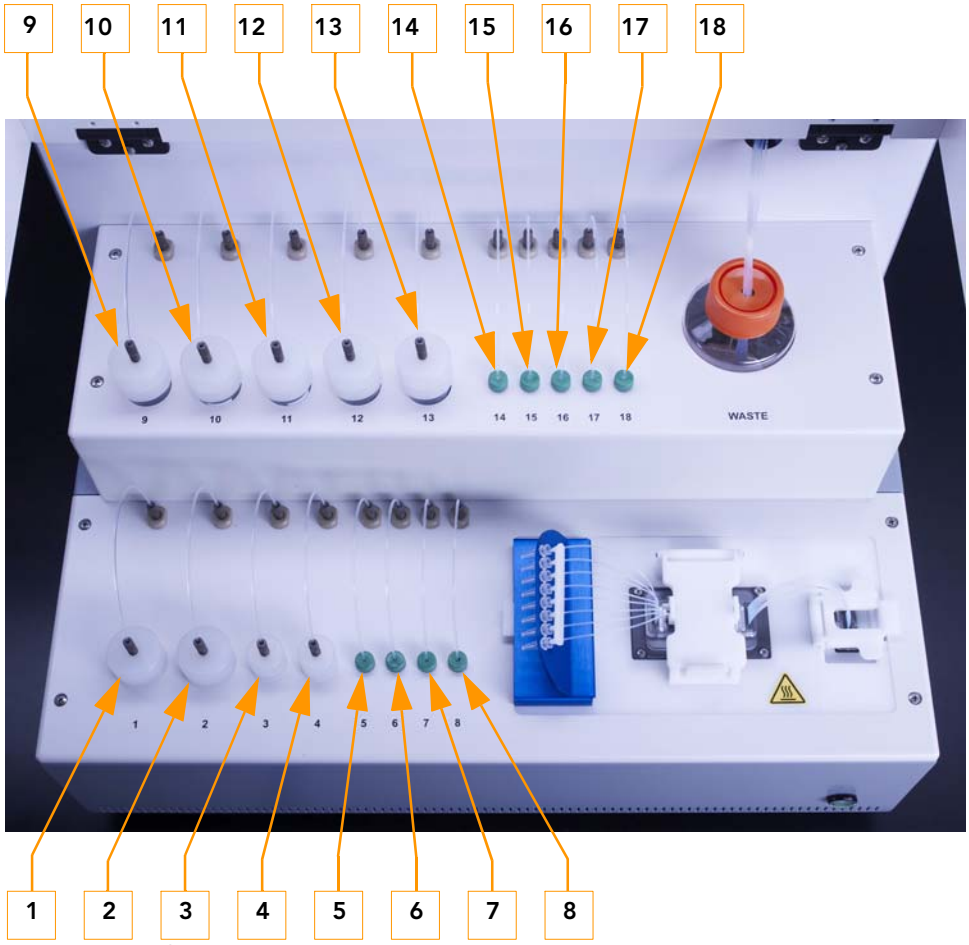


Figure 39 Cluster Station Reagent Positions

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

2. In the **Flowrate** field, 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 15–60 µl/min.

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

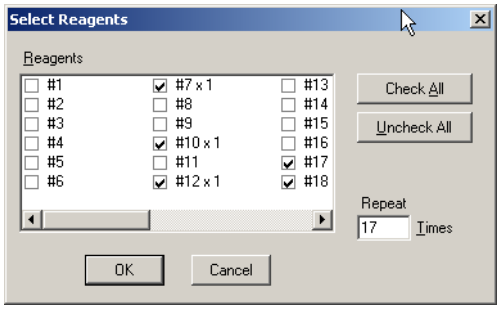


Figure 40 Select Reagents

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

   For reagent positions 5–8, 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.

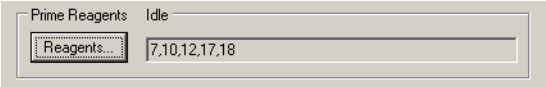


Figure 41 Lines Primed

Unclogging the Flow Cell

If the flow cell is blocked while priming with reagents, perform the following steps:

1. Remove the flow cell.
2. Put the flow cell back into the buffer.
3. Flick out any liquid from the manifold.
4. Reassemble as described in *Positioning the Flow Cell* on page 61.

Temperature Profile

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

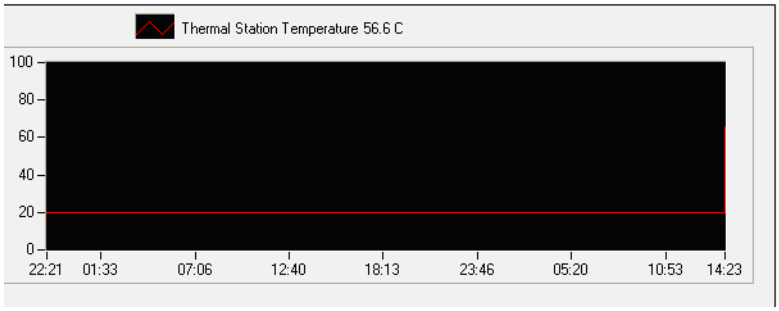


Figure 42 Temperature Profile

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:

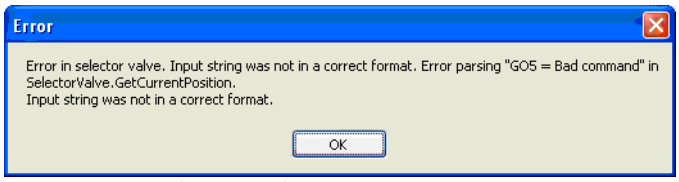


Figure 43 Selector Valve Error Message

To fix the problem:

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

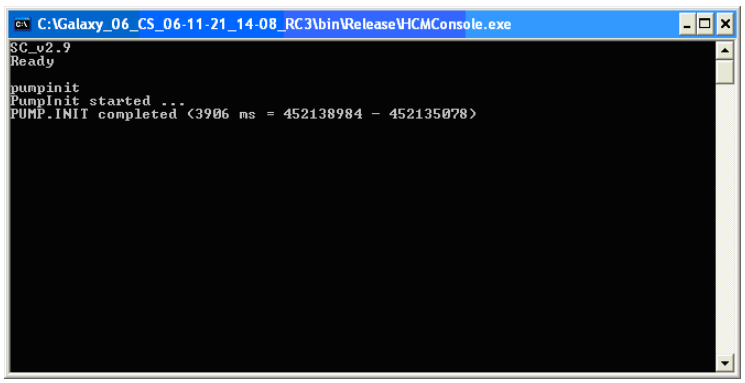


Figure 44 Pumpinit Command

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.

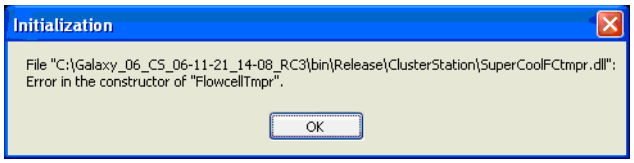


Figure 45 Flowcell Tmpr Error Message

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.

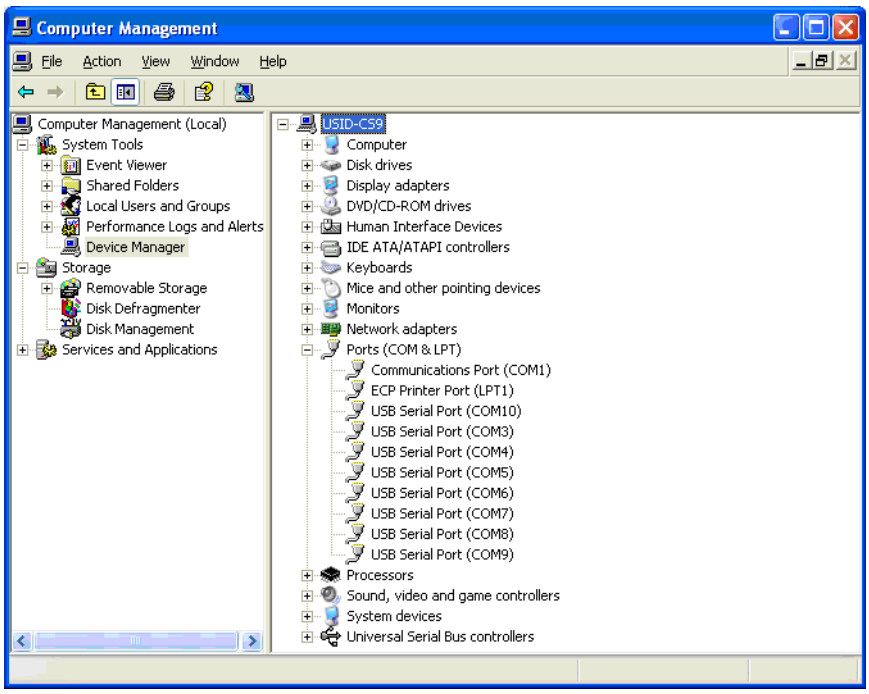


Figure 46 COM Port Settings in Device Manager

   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 4

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.

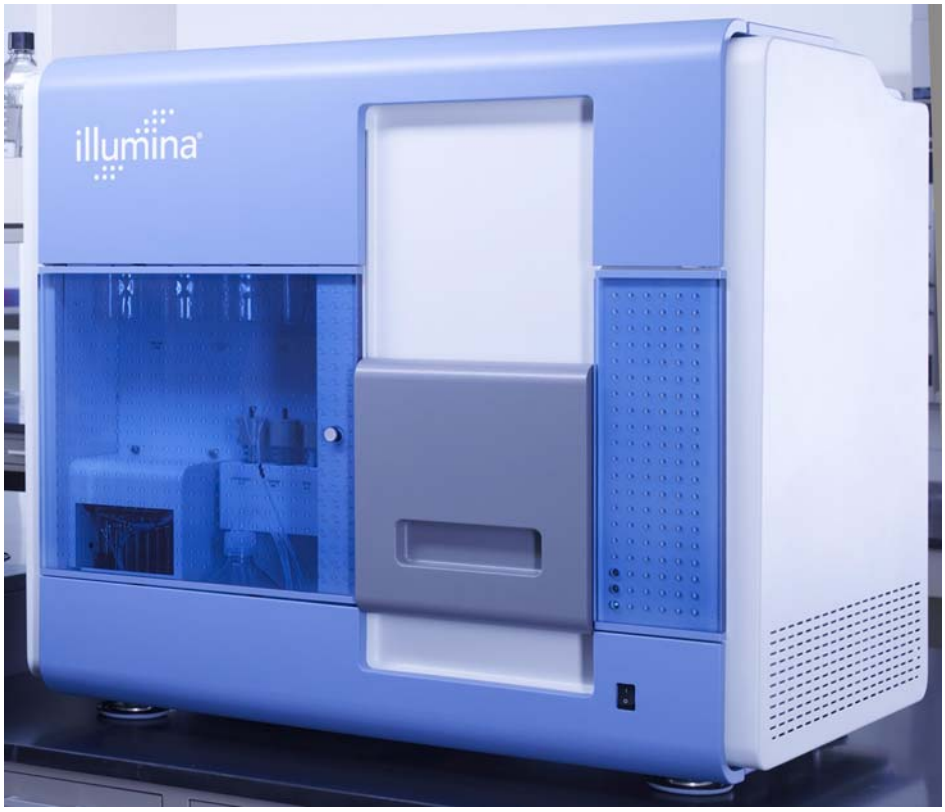


Figure 47 Genome Analyzer

Workflow

The following figure illustrates the workflow on the Genome Analyzer, and the reagents and components used in each step.

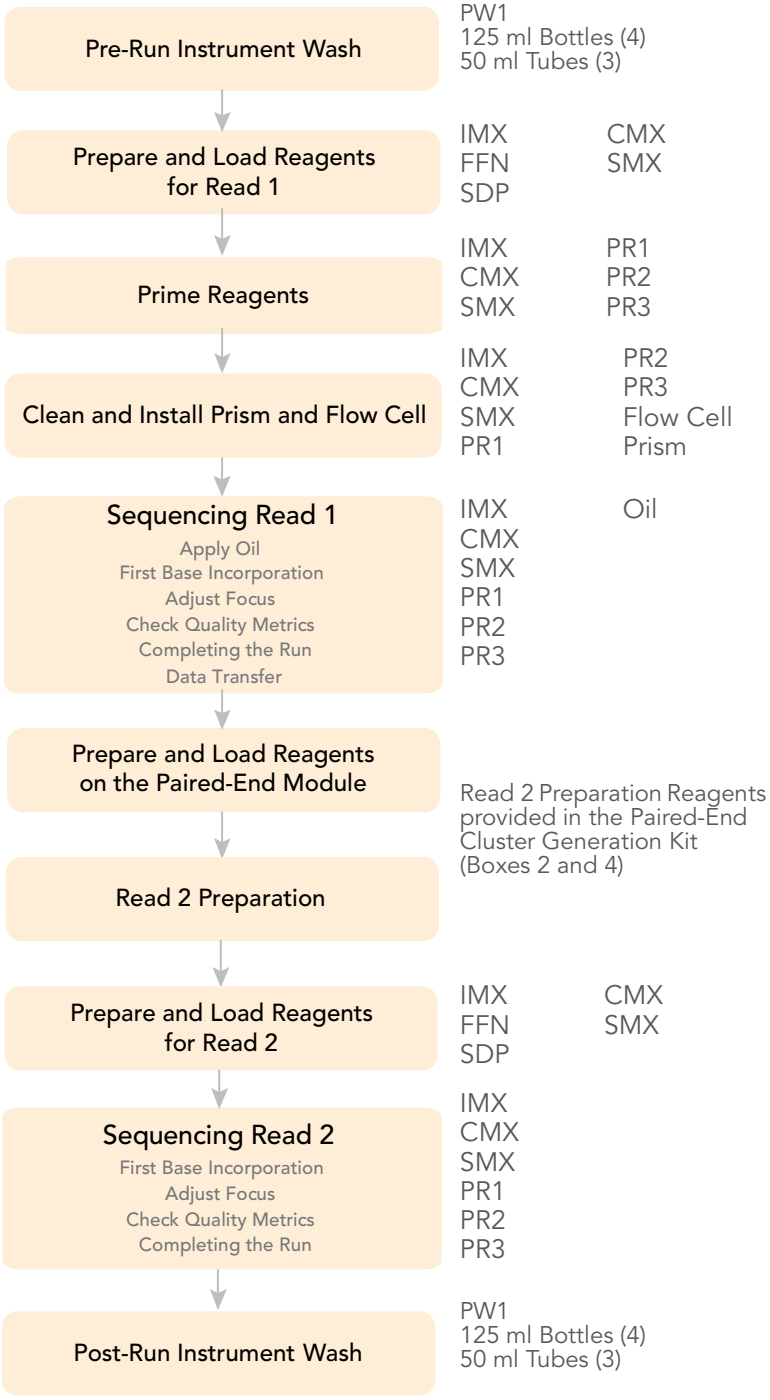


Figure 48 Paired-End Workflow on the Genome Analyzer

Procedures

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

1. *Starting the Genome Analyzer* on page 88
2. *Performing a Pre-Run Wash* on page 105
3. *Prepare Reagents for Read 1 on the Genome Analyzer* on page 101
4. *SBS Sequencing Kit v2 Contents* on page 99
5. *Loading and Priming Reagents* on page 107
6. *Cleaning and Installing the Prism* on page 110
7. *Cleaning and Installing the Flow Cell* on page 113
8. *Checking for Leaks and Proper Reagent Delivery* on page 117
9. *Applying Oil* on page 119
10. *Performing First-Base Incorporation* on page 121
11. *Loading the Flow Cell with Scan Mix* on page 123
12. *Adjusting Focus* on page 124
13. *Checking Quality Metrics* on page 136
14. *Completing Read 1* on page 139
15. *Data Transfer for Paired-End Runs* on page 140
16. *Preparing Reagents for Read 2 Preparation on the Paired-End Module* on page 141
17. *Preparing for Read 2 on the Paired-End Module* on page 148
18. *Preparing Reagents for Read 2 on the Genome Analyzer* on page 149
19. *Sequencing Read 2* on page 152
20. *Performing Post-Run Procedures* on page 155

Components

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

- Reagent Compartment
- Imaging Compartment

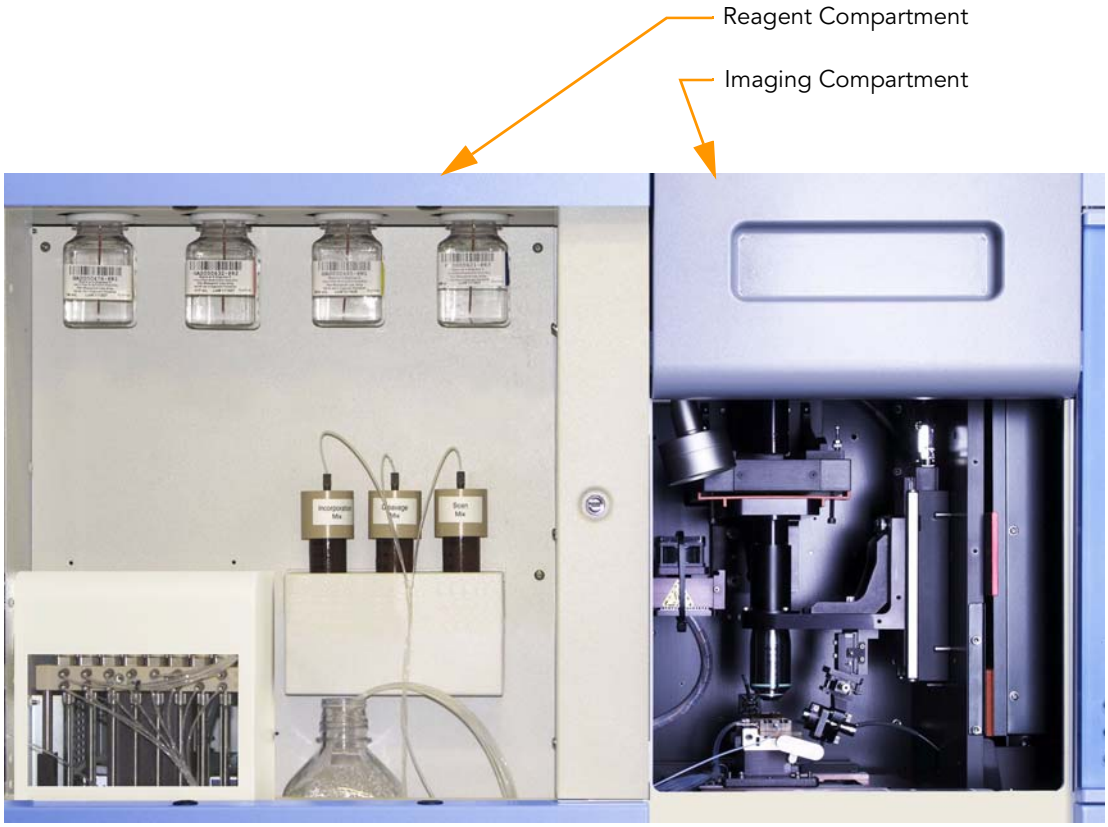


Figure 49 Genome Analyzer Main Compartments

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 fluidics pump.

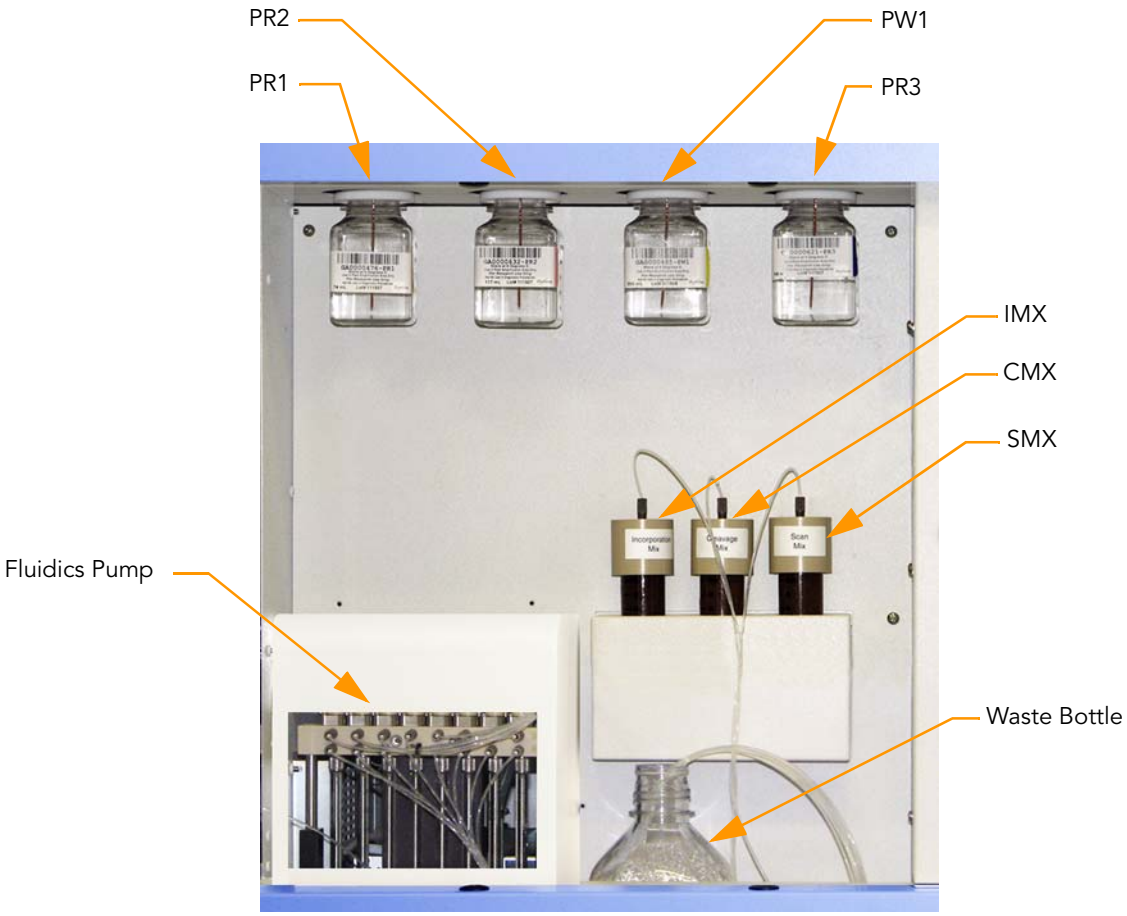


Figure 50 Genome Analyzer Reagent Compartment

Each reagent is numbered according to the position it connects to on the fluidic valve. The 125 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.

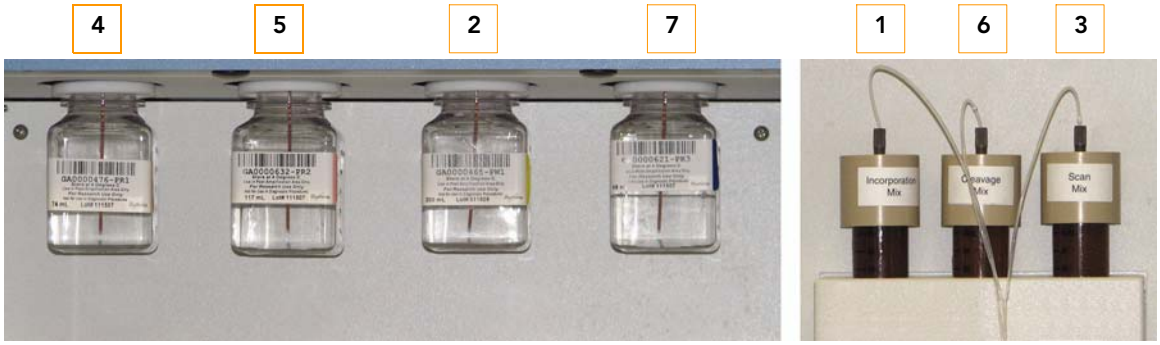


Figure 51 Reagent Positions

Table 11 Genome Analyzer Reagent Names

Solution #	Reagent Name	Reagent
1	IMX	Incorporation Mix
2	PW1	Deionized Water
3	SMX	Scan Mix
4	PR1	High Salt Buffer
5	PR2	Incorporation Buffer
6	CMX	Cleavage Mix
7	PR3	Cleavage Buffer

Imaging Compartment

The right-side compartment houses the Imaging Station, Thermal Station, Flow Cell Stage, Fiber Optics Mount, and Manifold.

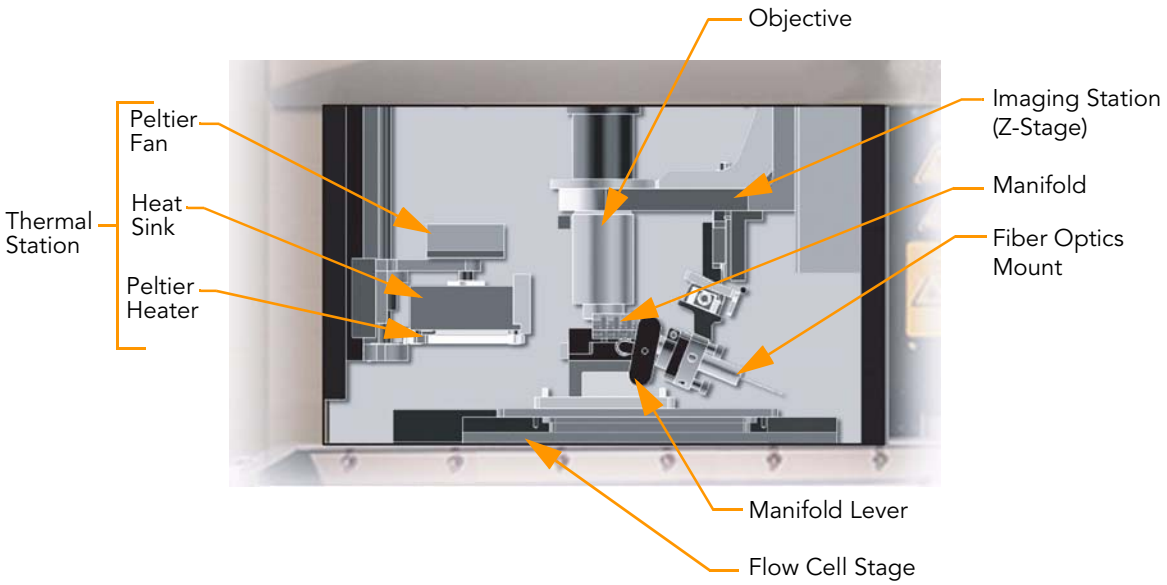


Figure 52 Genome Analyzer Imaging Compartment

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.

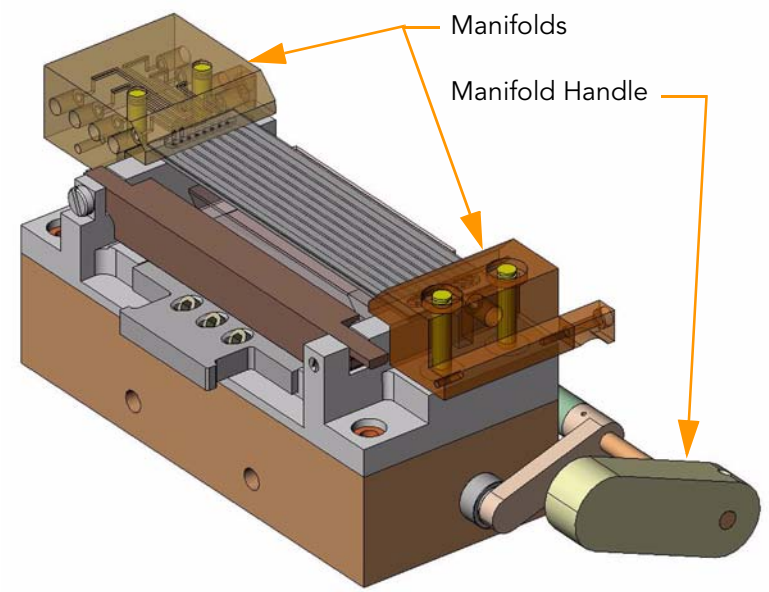


Figure 53 Front and Rear Plumbing Manifolds

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 SCS software 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 using the default values:

   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 Run folder on the D partition to hold the run data (if not automatically created). For more information, see Appendix A, *Run Folders*.

The Genome Analyzer *II* is set up to run 1.4 mm flow cells. Although it is possible to run 1.0 mm flow cells this requires a configuration change to the instrument that can only be done by a Technical Support representative (for contact information, see *Technical Assistance* on page 4).

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

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

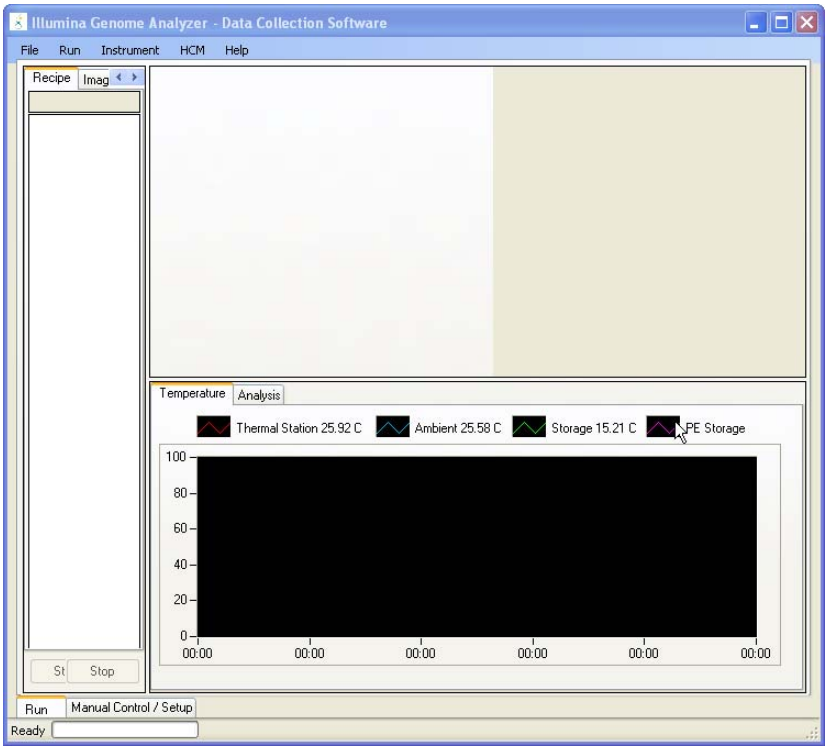


Figure 54 Genome Analyzer Software Screen

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 ignores inputs until the routine is complete.

Starting IPAR

1. Make sure the IPAR uninterruptible power supply (UPS) switch is in the ON position.
2. Turn the IPAR server power switch to the ON position.

   Before proceeding to the next step, wait until the IPAR server has fully started. This could take up to three minutes.
3. Log on to the IPAR server:
   a. Open the remote desktop application on the instrument computer by selecting **START | All Programs | Accessories | Remote Desktop Connection** from the task bar.
   b. Connect to 192.168.137.20.
   c. Log on using the default values:
   Username: sbsuser
   Password: sbs123

Network Copy

Images, log files, and other run files are copied automatically from the local drive to a network location while the run is proceeding. This saves a considerable amount of time transferring data upon completion of a run. For an explanation of this feature, or to change the network copy configuration, see *Network Copy Options* on page 190.

Software User Interface

This section describes the main windows, views, and controls of 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.

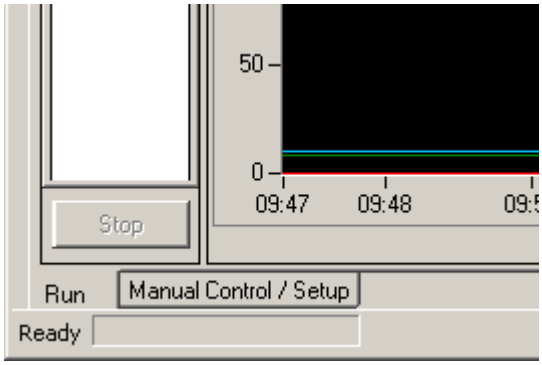


Figure 55 Run and Manual Control/Setup Windows

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.

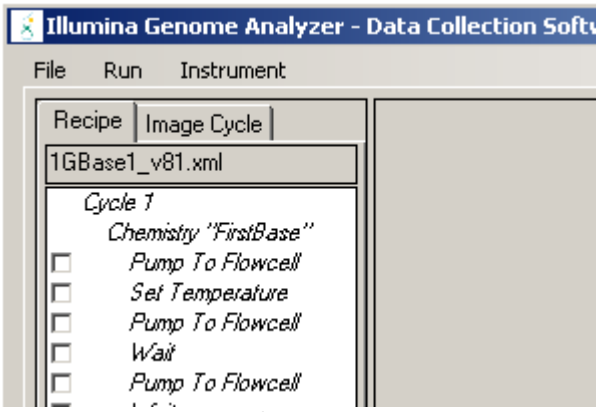


Figure 56 Recipe Tab

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

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

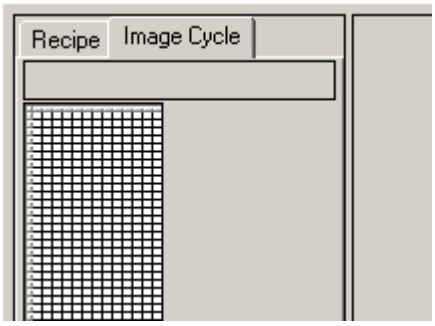


Figure 57 Image Cycle Tab

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

NOTE

The Genome Analyzer GUI has to be maximized to display all the tiles correctly.

Temperature and Analysis Viewer Tabs

Two tab views are available at the bottom of the Run window (Figure 58). By default, the Temperature tab is in view.

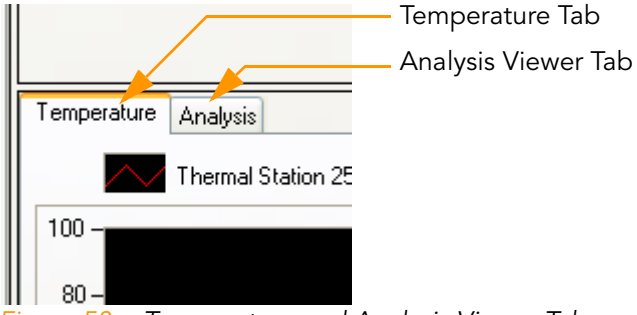


Figure 58 Temperature and Analysis Viewer Tabs

The Temperature tab lets you monitor various run temperatures.

The Analysis Viewer tab brings up the Analysis Viewer, a part of Integrated Primary Analysis and Reporting (IPAR). The Analysis Viewer displays key quality metrics in real time, so you can quickly decide whether or not to complete the run. To learn about IPAR and the Analysis Viewer, see Chapter 6, *Integrated Primary Analysis and Reporting*.

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 12 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 Genome Analyzer Software 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 current X and Y values of the stage.
See intensity values	Roll the mouse over a point of raised intensity to display the 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 12-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 to any area over the tile image in order to display the focal quality and uniformity.
Show region of interest	Right-click over the image and select **Show | ROI** to show the region of interest (ROI), which is the area that is saved during the sequencing runs. The ROI is indicated by the dotted lines on the display, and this feature allows you to check for good agreement of the ROI edge and lane edge.
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 59 Pump Control Area

Table 13 Pump Controls

Control	Description
Command	**Pump**—Transfers liquid from the port indicated in the **Solution** field to the location indicated in the **To** field. 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	The port from which the instrument will draw liquid. Note: Solution 28 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.

The Pump Control contains an additional tab for the manual operation of the Paired-End Module.

Basic Procedures

Washing the Lines

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

- *Performing a Pre-Run Wash* on page 105
- *Monthly Maintenance and Storage Wash* on page 95
- *Post-Run Wash* on page 155

Monthly Maintenance and Storage Wash

The Maintenance Wash has two parts: the 1 ml water wash and the 4 ml NaOH wash. Perform the Maintenance Wash once monthly for maintenance, and any time you plan to leave the Genome Analyzer sitting idle for more than three days. The monthly wash uses the same instrument cycle as the pre-run wash, but uses a stronger base (1 N NaOH) and filtered cleaning liquids.

Perform the storage wash if you plan to store the Genome Analyzer for more than three days.

Consumables

Illumina-Supplied

- PW1 (Wash Solution)

User-Supplied

- Lens cleaning tissue
- Nylon filter (0.2 µm pore size)
- MilliQ water for washing the Paired-End Module
- 1 N NaOH (0.5 L, filtered with a 0.2 µm nylon filter)
- 125 ml Nalgene bottles (4) for PW1 wash solution (ThermoFisher Scientific, catalog # 2019-0125)
- 50 ml conical tubes (3) for PW1 wash solution

Maintenance Wash

The Maintenance Wash should be performed once a month to wash the Genome Analyzer and Paired-End Module. The wash cycle runs for approximately 1 hour and 24 minutes.

1. 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. *Loading the Flow Cell* on page 113Load the clean, dry flow cell as stated in the *Loading the Flow Cell* on page 114.

When the manifolds are raised for the first time, place the fluidics on standby by performing steps 5 and 6 of *Unloading a Flow Cell* on page 97.

2. Select **File | Open Recipe**.
3. Open the GA2-PEM_MaintenanceWash_v<#>.xml recipe.

   The following prompt appears: "Please Load Wash Solutions onto the Genome Analyzer and Water onto the Paired-End Module. Press OK to Start Prewash."
4. Load the Genome Analyzer with PW1 wash solution as follows:

   10 ml for positions 1, 6, and 3

   40 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.

5. Load the Paired-End Module with 15 ml tubes containing 10 ml of MilliQ water on positions 9–21.
6. Click **OK** to start the prewash.

   When the prewash is complete, the following prompt appears: "Prewash Completed. Please Load 1N NaOH onto the Genome Analyzer and the Paired-End Module. Press OK to Start 1N NaOH Wash."
7. Load the Genome Analyzer with filtered 1 N NaOH as follows:

   25 ml for positions 1, 6, and 3

   50 ml for port positions 4, 5, 2, and 7
8. Load the Paired-End Module with 15 ml tubes containing 10 ml of filtered 1 N NaOH on positions 9–21.
9. Click **OK** to start the NaOH wash.

   After the NaOH wash is complete, the following prompt appears: "NaOH Wash Completed. Please Load Wash Solutions onto the Genome Analyzer and Water onto the Paired-End Module. Press OK to Start Post-wash."
10. Load the Genome Analyzer with PW1 wash solution as follows:

    15 ml for positions 1, 6, and 3

    50 ml for port positions 4, 5, 2, and 7
11. Load the Paired-End Module with 15 ml tubes containing 15 ml of MilliQ water on positions 9–21.
12. Click OK to start the post-wash.

    When the post-wash is complete, the following prompt appears: "Monthly Maintenance and Storage Wash Completed."

Storage Wash

If you plan to leave the Genome Analyzer idle for more than three days, perform this wash after the maintenance wash.

1. Load wash solutions into port positions 1, 6, 3, 4, 5, and 7 on the Genome Analyzer.

   Position 2 remains loaded with water.

2. Place at least 5 ml of MilliQ water in each Falcon tube in positions 9–21 on the Paired-End Module.
3. Remove any tubing connected to port position 8 and close the port with the appropriate stopper.
4. Click the Run tab.
5. Select **File | Open Recipe**.
6. Open the GA2-PEM_PostWash_v<#>.xml wash recipe file.
7. Click **Start**.
8. When the run finishes, click the Manual Control tab.
9. In the Pump area, set the parameters as follows:
   Solution: 28
   Volume: 0
10. With the cursor in the **Volume** field, press **Enter**.
11. Leave the flow cell in the instrument to prevent siphoning.
12. Close the Genome Analyzer software and shut down the computer.
13. Turn the Genome Analyzer power switch to the OFF position.

Resuming Use after Storage

Perform the following steps to resume instrument use after short-term 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 on the Genome Analyzer.
5. Load 0.5 L filtered, deionized water into Position 2.
6. Place at least 5 ml of MilliQ water in each Falcon tube in positions 9–21 on the Paired-End Module.
7. Click the Run tab.
8. Select **File | Open Recipe**.
9. Open the GA2-PEM_PreWash_v<#>.xml wash recipe.
10. Click **Start**.

Unloading a Flow Cell

Perform the following steps to unload a flow cell from the stage:

1. Click the Manual Control/Setup tab.
2. Click **Load Flowcell** to slide the stage forward.
3. Select **Instrument | Unlock Door** to release the door to the imaging compartment.
4. Click the Manual Control/Setup tab.

5. In the Pump area, make sure the following values are set:
   Command: Pump
   To: Flowcell
   Solution: 28 (to prevent siphoning reagents)
   Volume: 0
   Aspiration Rate: 250
   Dispense Rate: 2500
6. With the cursor in the **Dispense Rate** box, press **Enter**.
7. Turn the manifold handle clockwise to lift the manifolds.

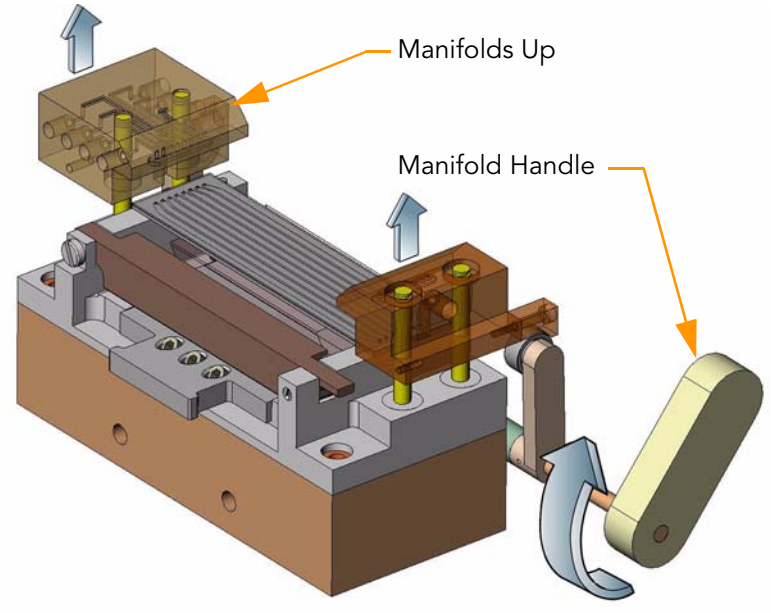


Figure 60 Lifting Front and Rear Manifolds

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

SBS Sequencing Kit v2 Contents

This section describes the features and contents of 36-Cycle SBS Sequencing Kit v2. For information regarding the 26-Cycle Sequencing Kit, see *Preparing Reagents for the Genome Analyzer.*

What's New

- The hands-on time required to prepare reagents for the Genome Analyzer is reduced from about 90 minutes to 10 minutes.
- The only reagent that needs to be prepared prior to loading onto the Genome Analyzer is the IMX, which is made by combining all the contents of the FFN and SDP tubes into the IMX container.
- No filtering of the reagents is required.
- The reagents are provided in containers that load directly onto the instrument. The bottles are shatter-resistant and provide excellent gas barrier properties for storage and shipping of buffers.
- Four bottle adaptors are required for position 4, 5, 2, and 7 to hold the new shatter-resistant reagent bottles.
- The kit is condensed into a single package containing two boxes that are shipped on dry ice. Once the package arrives, one box is stored at 4°C and the other box at -20°C.
- Reagent labels are color coded to help reduce the possibility of reagent mix-up while loading the reagents. Both the reagent boxes and reagent containers include a barcode ID enabling reagent tracking.
- The IMX, SMX, and CMX are provided in a dark amber tube to better protect and preserve the reagents.
- The reagent names have changed. The following table lists the Genome Analyzer reagents and the reagent names in the SBS Sequencing Kit v2. Each reagent name contains a prefix of GA# and a suffix based on the number of cycles you are running.

Table 14 Genome Analyzer Reagents

Position #	Reagent Name	Reagent
1	IMX	Incorporation Mix (IMX, FFN, and SDP)
2	PW1	Deionized Water
3	SMX	Scan Mix
4	PR1	High Salt Buffer
5	PR2	Incorporation Buffer
6	CMX	Cleavage Mix
7	PR3	Cleavage Buffer

Check to ensure that you have all of the reagents identified in this section before proceeding to Read 1 on the Genome Analyzer.

Reagents and other consumables are shipped separately from the instrument. They are boxed according to storage temperature requirements.

It is very important to promptly store the reagents at the temperature specified on the box to ensure that they perform correctly.

All reagents included in the SBS Sequencing Kit v2 are labeled with the prefix GA#.

SBS Sequencing Kit, Box 1

Store at 2° to 8°C

This box is shipped on dry ice. When you receive your kit, store the components at 2° to 8°C. If you plan to use the components the next day, thaw them at room temperature overnight.

- PW1
- PR1
- PR2
- PR3

SBS Sequencing Kit, Box 2

Store at -15° to -25°C

This box is shipped on dry ice. When you receive your kit, store the components at -15° to -25°C.

- IMX36
- FFN36
- SDP36
- SMX36
- CMX36

Prepare Reagents for Read 1 on the Genome Analyzer

Follow these instructions to prepare reagents before loading them onto the Genome Analyzer. Required materials are provided in the 36-Cycle Sequencing Kit v2.

When you prepare and load reagents onto the Genome Analyzer, you must use them in a sequencing run the same day.
Exception: The High Salt Buffer does ***not*** need to be made fresh for each sequencing run.

Unpack and Thaw Reagents

1. Remove the following reactive part components from -20°C storage and thaw them at room temperature or in a beaker containing deionized water. Do not microwave.
   - IMX36
   - FFN36
   - SMX36

   If you use the beaker method, make sure the water line does not reach the cap of the tube to prevent contamination.

   Leave the SDP36 in -20°C storage until you are ready to use it to make the Incorporation Mix.

It is important to keep the CMX away from the other components to avoid cross-contamination.

2. Remove the CMX36 from -20°C storage and thaw it at room temperature or in a ***separate*** beaker containing deionized water. Do not microwave. If you use the beaker method, make sure the water line does not reach the cap of the tube to prevent contamination.

After handling the CMX container, be sure to discard your gloves and replace them with a new pair each time.

3. Record the lot numbers of each reagent on the lab tracking worksheet.
4. Immediately after the reagents have thawed, place them on ice. Be sure to keep the CMX36 in a ***separate*** ice bucket during reagent preparation.
5. If the components from Box 1 are still frozen, thaw them in a container of deionized water.

Procedure

IMX36

Required Materials:

- FFN36
- IMX36
- SDP36

1. Transfer 1.75 ml of the FFN36 into the IMX36.
2. Remove the SDP36 tube from -20°C storage and briefly pulse centrifuge.
3. Transfer 220 µl of SDP36 to the IMX36 reagent (containing FFN36).
4. Cap the IMX36 (containing FFN36 and SDP36) tube tightly and invert five times to mix.
5. Centrifuge at 1,000 xg for 1 minute at 22°C.
6. Place the IMX36 on ice until you are ready to load it onto the Genome Analyzer.
7. Record the weight of the reagent in the lab tracking worksheet.

PR1

1. Invert the bottle of PR1 several times before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

PR2

1. Invert the bottle of PR2 several times to mix before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

PR3

1. Invert the bottle of PR3 several times before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

SMX36

1. Invert the SMX36 tube several times to mix well, and then centrifuge at 1,000 xg for 1 minute at 22°C before loading it onto the Genome Analyzer.
2. Record the weight of the reagent in the lab tracking worksheet.
3. Place the SMX36 on ice until ready to load onto the Genome Analyzer.

CMX36

1. Invert the CMX36 tube several times to mix well, and then centrifuge at 1,000 xg for 1 minute at 22°C before loading it onto the Genome Analyzer.
2. Record the weight of the reagent in the lab tracking worksheet.

3. Place the CMX36 in a ***separate*** ice bucket until you are ready to load it onto the Genome Analyzer.
4. Discard your gloves and replace them with a new pair.

When you load the reagents onto the Genome Analyzer, load the CMX last to avoid cross-contamination.

Be sure to perform a pre-run wash before loading reagents onto the Genome Analyzer.

PW1

1. To prepare for the Genome Analyzer pre-run wash, aliquot 40 ml of PW1 into four 125 ml Nalgene bottles.
2. Aliquot 10 ml of PW1 into three 50 ml conical tubes.

See *Performing a Pre-Run Wash* on page 105 for pre-run wash instructions.

Installing the Bottle Adaptors

1. Unpack four Genome Analyzer bottle adaptors.

Figure 61 Genome Analyzer Bottle Adaptor

2. Install the bottle adaptors at positions 4, 5, 2, and 7 on the Genome Analyzer by threading the adaptor completely into the instrument. Hand-tighten each adaptor until the rim of the adaptor is barely visible.

NOTE

Do not overtighten the adaptors.

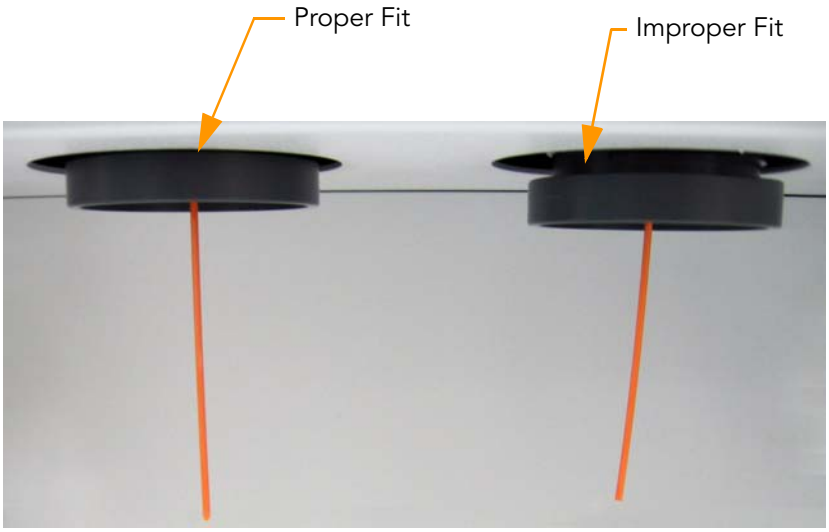


Figure 62 Proper Fit of Bottle Adaptor

Performing a Pre-Run Wash

You must perform a pre-run wash if the instrument has been idle for one day or more, and before changing and priming reagents. The wash flushes 1 ml of instrument wash reagent (PW1) through each reagent port and out to a waste container. Run time is approximately 15 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)
- MilliQ water for washing the Paired-End Module
- 125 ml Nalgene bottles (4)
  (ThermoFisher Scientific, catalog # 2019-0125)
- 50 ml conical tubes (3)

Illumina-Supplied

- PW1

Procedure

1. Load the instrument with a used flow cell. See *Loading the Flow Cell* on page 114.
2. Dispense 40 ml of PW1 into four 125 ml Nalgene bottles.
3. Dispense 10 ml of PW1 into three 50 ml conical tubes.
4. Load the instrument with solutions as follows:
   - 10 ml PW1 into port positions 1, 6, and 3
   - 40 ml PW1 into 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.

5. Place at least 5 ml of MilliQ water in each Falcon tube in positions 9–21 on the Paired-End Module.
6. Loosen and remove the waste tubing.
7. Bundle all waste tubes with parafilm, making sure to keep all of the ends even.
8. Place the bundled tube ends into a 50 ml tube.
9. Click the Run tab.

10. Select **File | Open Recipe**.
11. Open the GA2-PEM_PreWash_v<#>.xml recipe.

    This recipe washes the Genome Analyzer and the Paired-End Module.
12. Click **Start**.

    The wash cycle runs for approximately 40 minutes. Reagents are delivered 1 ml at a time. You should collect a total volume of 21 ml.
13. 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.

During the sequencing run, keep one 125 ml Nalgene bottle containing PW1 at position 2 on the Genome Analyzer.
Save the 125 ml bottles and the 50 ml conical tubes containing the PW1 solution for use with the post-run wash.

Loading and Priming Reagents

Always perform a pre-run wash before loading reagents onto the Genome Analyzer. See *Performing a Pre-Run Wash* on page 105.

Loading Reagents

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

Safe Handling Conventions

1. To prevent cross-contamination of reagents, especially the IMX and CMX, establish safe handling conventions such as:
   - Always remove and replace one bottle or tube at a time.
   - Always install the CMX *last* to avoid cross-contamination.
   - Keep the SMX, IMX, and CMX on ice until you load them onto the Genome Analyzer.
2. Invert all reagents several times to mix them before loading them onto the Genome Analyzer.
3. Centrifuge the SMX, CMX, and IMX at 4°C at 1000 xg for 1 minute before loading them onto the Genome Analyzer.

Reagent Positions

Load the prepared reagents in the appropriate positions on the Genome Analyzer, as shown in the following figure and corresponding table. When you attach the 50 ml tubes, hold the caps stationary and rotate the tubes to prevent crimps in the liquid delivery lines.

NOTE

Save the 125 ml bottles and the 50 ml conical tubes containing the PW1 solution for use with the post-run wash.

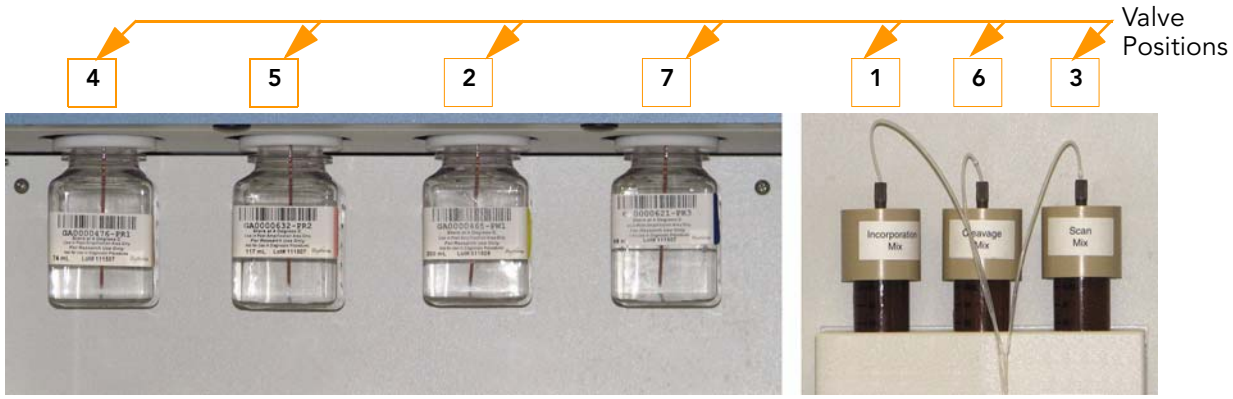


Figure 63 Genome Analyzer Reagent Positions

Table 15 Genome Analyzer Reagent Positions

Solution #	Size	Contents
1	50 ml Amber Tube	IMX36
2	125 ml Bottle	PW1
3	50 ml Amber Tube	SMX36
4	125 ml Bottle	PR1
5	125 ml Bottle	PR2
6	50 ml Amber Tube	CMX36
7	125 ml Bottle	PR3

Automatically tracking reagents by reading in the barcodes for the reagents is not available for paired-end sequencing.

Priming Reagents

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.

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.

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 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 GA2_Prime_v<#>.xml recipe.
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:

a. Click the Manual Control/Setup tab. In the Pump area, set the following values:
Command: Pump
To: Flowcell
Solution: 28
Volume: 0
Aspiration Rate: 250
Dispense Rate: 2500

b. Click **Load Flow Cell** to bring the stage to the front of the instrument and raise the lens.

c. In the **Instrument** pull down menu, select **Unlock Door**. Raise the door.

d. Lift the manifolds and reposition the flow cell.

The manifolds should only be lifted for a small period of time, else the manifolds will back flush onto the flow cell.

e. Repeat the priming procedure.

10. Proceed to *Cleaning and Installing the Prism* on page 110.

Cleaning and Installing the Prism

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

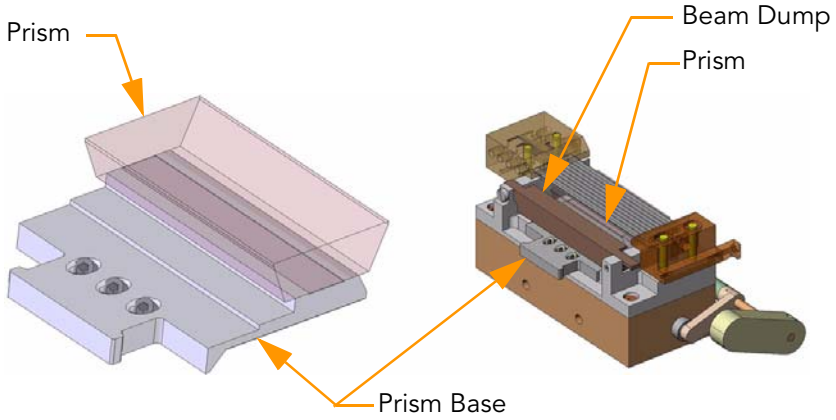


Figure 64 Prism

Handling the Prism

Always wear powder-free latex gloves when handling the prism. Hold the prism by the metal prism base only.

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

Removing the Flow Cell and Prism

1. Click **Load Flow Cell** to bring the stage to the front of the instrument and raise the lens. When the Flow Cell ID dialog box displays, click **Cancel**.
2. In the **Instrument** pull down menu, select **Unlock Door**. Raise the door.
3. Click the Manual Control/Setup tab.
4. In the Pump area, make sure the following values are set:
   Command: Pump
   To: Flowcell
   Solution: 28 (to prevent siphoning reagents)
   Volume: 0
   Aspiration Rate: 250
   Dispense Rate: 2500
5. With the cursor in the **Dispense Rate** box, press **Enter**.
6. Turn the manifold handle clockwise to lift the manifolds.

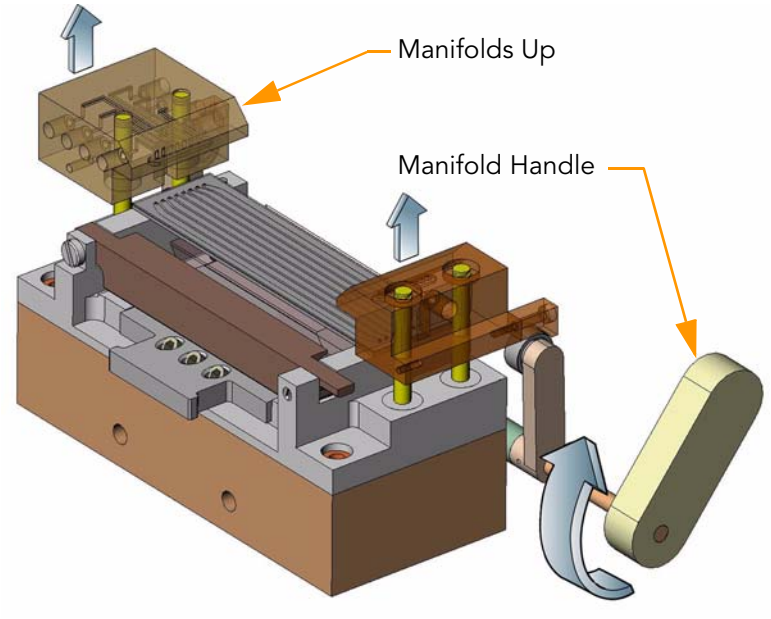


Figure 65 Lifting Front and Rear Manifolds

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

Cleaning the Prism

Consumables

User-Supplied

- Lint-free lens cleaning tissue
- 100% ethanol or Spectrophotometer-grade methanol

1. Put on new gloves.
2. Wipe down any oil that has spilled onto the flow cell holder or accumulated onto the beam dump.
3. Place the prism on a fresh ethanol wipe on the benchtop.
4. Remove the oil by gently washing the prism with a stream of ethanol or methanol.
5. Wipe the metal prism base with a lens cleaning tissue.
6. Fold a lens cleaning tissue to approximately the size of the prism. Wet the edge of the tissue with ethanol or methanol and wipe off the surface with a single sweeping motion. Repeat, refolding the tissue with each wipe, until the prism is completely clean.

CAUTION Be sure to remove any lint that is present on the prism or the flow cell.

7. 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.
8. Protect the prism from dust until you place it onto 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 or methanol.
2. Wipe the recessed surface of the prism holder to remove oil that may have been spilled during the previous run.
3. Lift the beam dump and slide in the prism assembly (Figure 66).

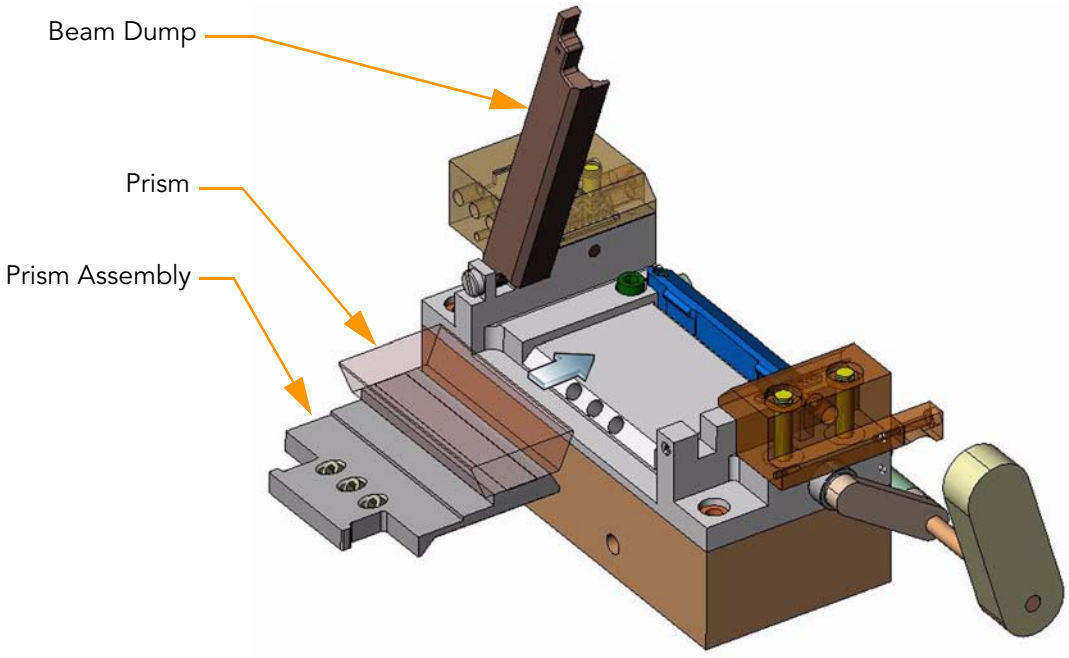


Figure 66 Loading the Prism

4. With prism in place, lower the beam dump.
   The prism assembly and beam dump lock in position.

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.

5. Proceed to *Cleaning and Installing the Flow Cell* on page 113.

Cleaning and Installing the Flow Cell

The flow cell is located above the prism and rests on the manifold mounting rails.

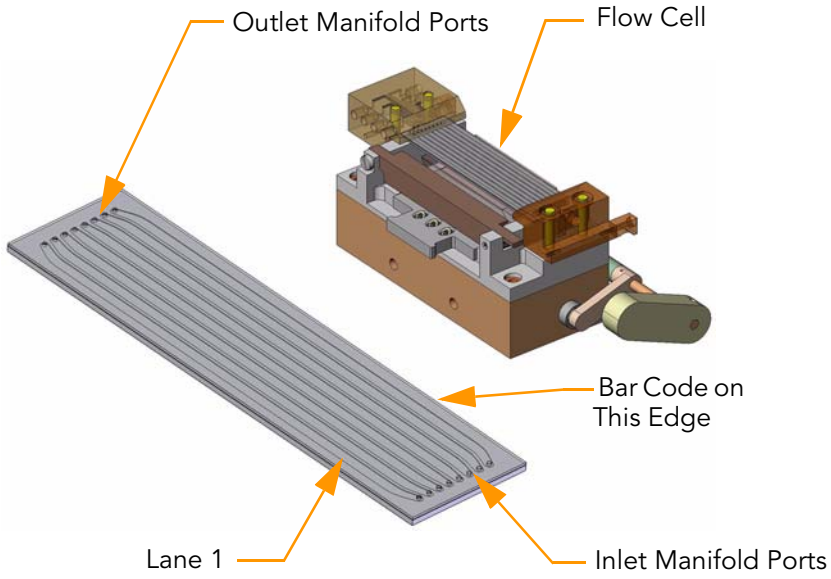


Figure 67 Flow Cell

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.

CAUTION If you clean the flow cell while it is lying on the bench top, you could easily apply too much pressure, and may break the flow cell. We recommend cleaning the flow cell while holding the edges between your 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 onto the Genome Analyzer.

Entering the Flow Cell ID

The Genome Analyzer can capture and save the bar code with the flow cell ID. A flow cell ID can also be typed by hand as an alternative to using the barcode reader.

Place the flow cell on a clean white background when reading the barcode.

1. Click **Load Flowcell** on the manual control screen, the stage moves to the load position, and the dialog box is displayed for the flow cell ID.
2. If you select **Cancel**, the dialog is closed, and no data changes are made (any previously entered flow cell ID remains current).
3. If you select **OK**, the ID field is checked to ensure that it isn't blank. If it is blank, a warning is displayed, and the operator must enter something or else cancel the dialog.
4. The operator can enter any characters from the keyboard. The barcode IDs are validated using the validation expression in the RCMConfig.xml file (FlowCellID= "[F][C][0-9][0-9][0-9][0-9]").

You can change the validation expression as required. In order to disable the barcode validation enter "" or empty string in the <FlowCellID> field.

5. Proceed to *Loading the Flow Cell* on page 114.

In some cases, for example after running a new recipe on a previously abandoned run, tracking of the flow cell ID may impede using the same flow cell. In such a case, you can set the <IgnoreFlowCellIDCheck> field in the RCMConfig.xml file to "true" to ignore the flow cell ID tracking.

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 right stops (Figure 68).

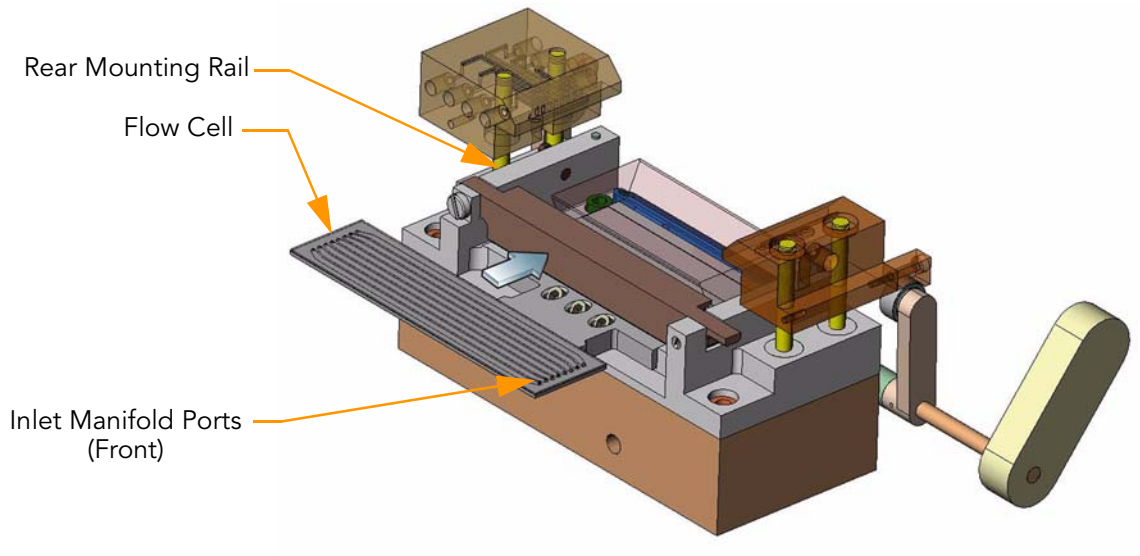


Figure 68 Loading the Flow Cell

2. Slide the flow cell to the back until you encounter the rear stop (Figure 69).

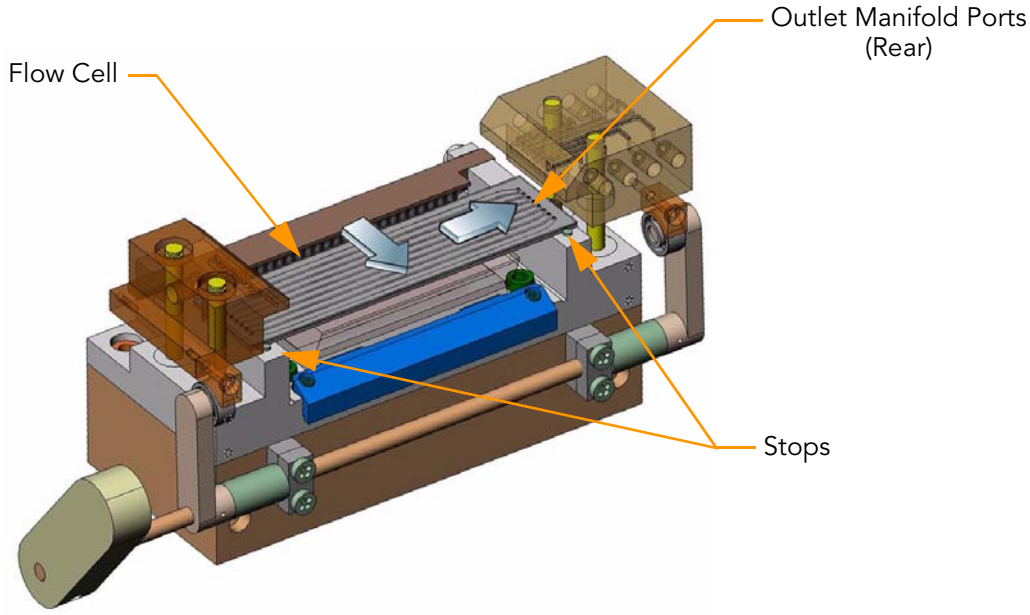


Figure 69 Positioning the Flow Cell

3. Using a lens cleaning tissue, gently apply pressure on the underside of the front manifold to absorb excess liquid.
4. Test proper placement by applying gentle pressure to the rear, then to the right to ensure the flow cell is pressed against both stops.
5. While holding the flow cell against the stops with one hand, carefully rotate the manifold handle counterclockwise with the other hand to lower the manifolds into place (Figure 70).

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.

6. Press downward on both manifolds to ensure they have seated properly (Figure 71).

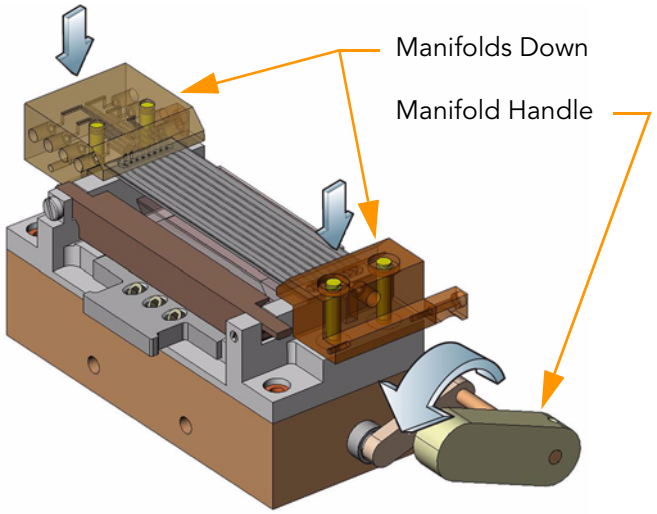


Figure 70 Lowering the Manifold

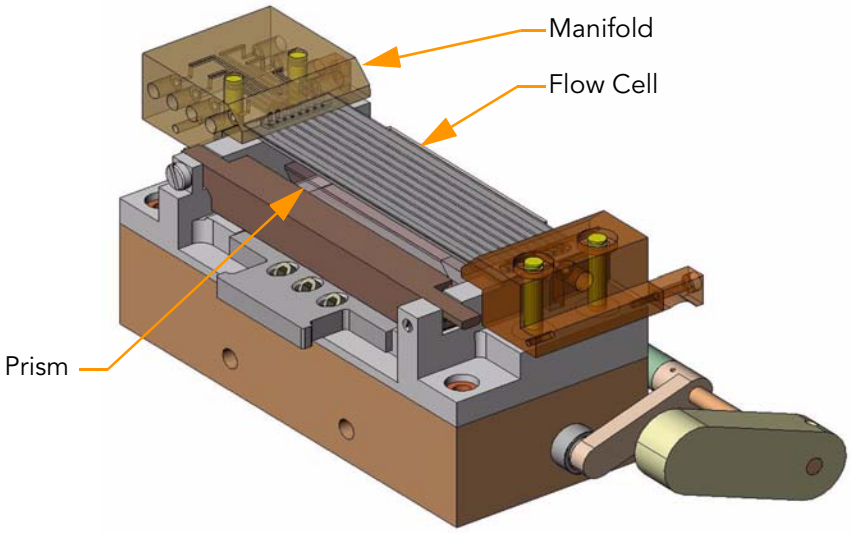


Figure 71 Flow Cell and Prism Loaded

Checking for Leaks and Proper Reagent Delivery

Reagent delivery volumes during the 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 Incorporation Buffer 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.
4. Pump 100 µl of Incorporation Buffer (solution 5) through the flow cell.
   a. Click the Manual Control/Setup tab.
   b. In the Pump area, set the values as follows:
      Command: Pump to Flow Cell
      To: Flowcell
      Solution: 5
      Volume: 100
      Aspiration Rate: 250
      Dispense Rate: 2500
   c. With the cursor in the **Dispense Rate** box, press **Enter**.
5. 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.

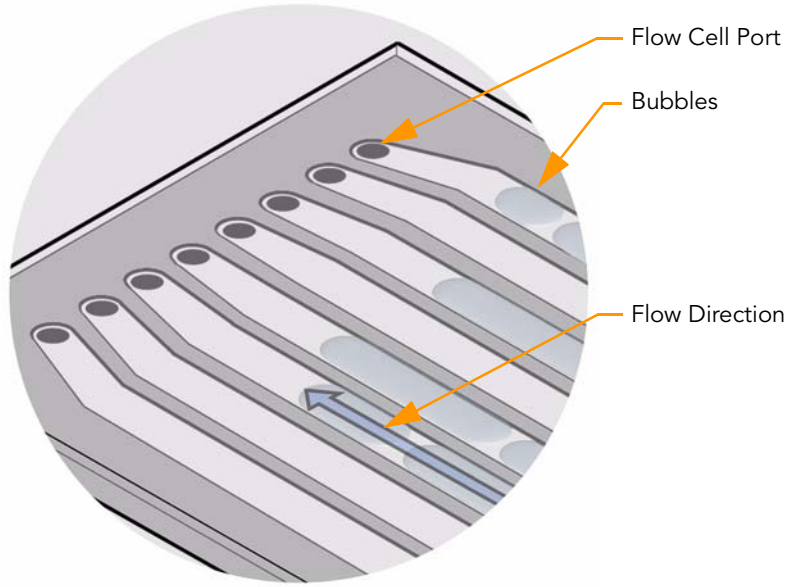


Figure 72 Checking for Bubbles

6. 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.

7. Check for leaks where the flow cell touches the manifold using a lens-cleaning tissue.

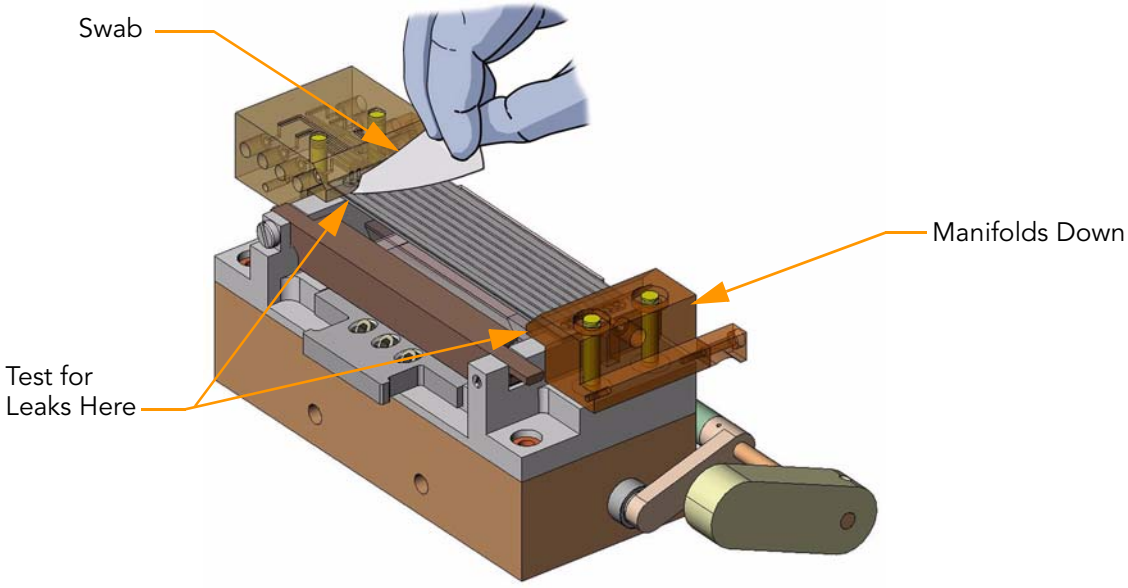


Figure 73 Testing for Leaks

If a leak is present, perform the following steps:

a. Click the Manual Control/Setup tab. In the Pump area, set the values as follows:
Command: Pump to Flow Cell
To: Flowcell
Solution: 28 (to prevent siphoning reagents)
Volume: 0
Aspiration Rate: 250
Dispense Rate: 2500

b. Click **Load Flow Cell** to bring the stage to the front of the instrument and raise the lens.

c. Lift the manifolds, clean the interface between manifold and flow cell with a moist lens tissue.

d. Dry and re-seat the flow cell.

e. In the Pump area, set the values as follows:
Command: Pump to Flow Cell
To: Flowcell
Solution: 5
Volume: 100
Aspiration Rate: 250
Dispense Rate: 2500

f. Pump another 100 µl of the Incorporation Buffer (solution 5) through the system.

8. 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 volume by more than 10%, have the instrument checked by an Illumina Field Service Engineer.

Once the system is leak free, the system is ready to run.

9. Proceed to *Applying Oil*.

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. Too much oil may result in images that are out of focus.

The amount of oil required varies from instrument to instrument. Based on mechanical tolerances, the amount of oil required will be between 70 and 125 µl. It should be fairly repeatable for each instrument and fixed prism pair, provided that the fixed prism and flow cell have been loaded properly.

1. Aspirate 135 µl of oil into the pipette, 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.

You may not need to dispense all of the immersion oil.

2. Place the pipette tip on the prism at the gap between the top surface of the prism and the front-left side of the flow cell, about 1 cm from the inlet manifold. Use two hands, with one hand on the tip to support and guide the tip.

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.

3. Dispense the oil slowly from the left side; dispensing too fast will result in oil on the top of the flow cell. Let all of the dispensed oil wick between the flow cell and the prism as far as it will go before dispensing more.

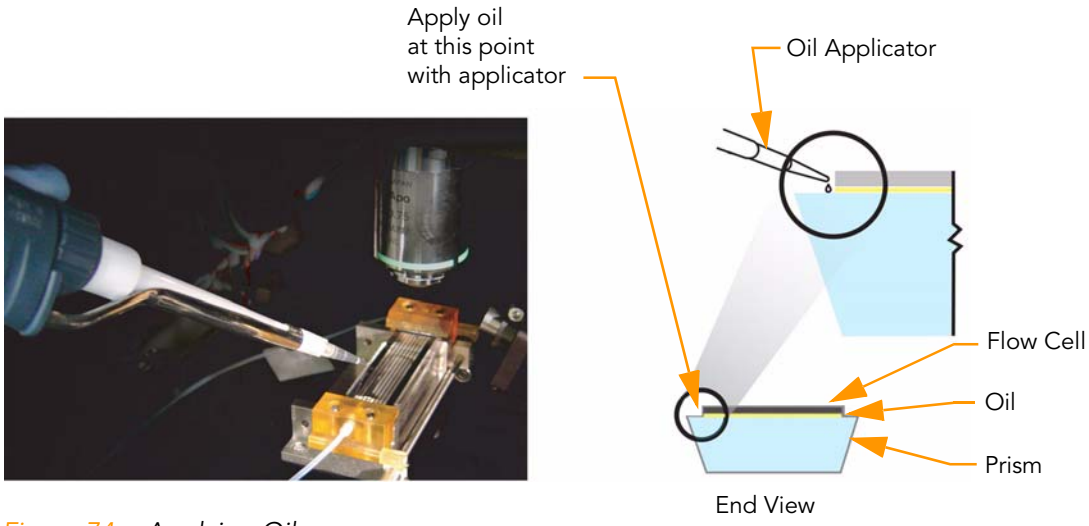


Figure 74 Applying Oil

4. Before the oil reaches the right side of the flow cell, slide the pipette tip in small steps towards the rear, steadily dispensing more oil along the way. Let all of the dispensed oil wick between the flow cell and the prism as far as it will go before dispensing more. The pipette tip must not move faster than the leading edge of the oil wicking under the flow cell.

   Observe the movement of the oil. Ensure there are no bubbles forming between the flow cell and the prism.

5. Stop moving the pipette when the tip is about 1 cm from the rear manifold.

   You have dispensed enough oil when it has wicked to the right edge of the flow cell.

CAUTION

Underloading the oil will cause a loss of illumination (most likely in lane 8, column 2, and nearest the inlet and outlet). Overloading could cause oil to wick over the imaging surface during the course of the run or spill over the right surface of the prism, both of which will cause problems when focusing images. Wicking may happen immediately, or later during a run when the oil heats up.

6. Ensure that the gap underneath the flow cell has a uniform layer of oil, that no bubbles exist between the flow cell and the prism, that there is no oil on the top of the flow cell, and that the right surface of the prism is clean.

   If anything is unsatisfactory, remove the flow cell and prism, clean them both thoroughly with alcohol wipes, reload, and repeat the application.

7. Use an ethanol wipe to clean the bottom surface of the Peltier heater. This ensures that no splashed immersion oil will be stamped on the flow cell by the Peltier.

8. Close the instrument door.

9. Proceed to *Performing First-Base Incorporation* on page 121.

Performing First-Base Incorporation

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

From this point on in the paired-end sequencing protocol, you can use either the two-folder paired-read method or the single-folder paired-read method. Each uses a different set of recipes. Both methods are described in each section.

The single-folder paired-read method uses a single recipe that performs a full 2 x 36 paired-end sequencing run and places the data in a single run folder.

Ensure that you are either running IPAR, RoboMove, or have sufficient hard drive space to accommodate two 36-cycle runs.

To use the single-folder paired-read recipe, you must be running SCS 2.01 or later.

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] Depending on the method you are using, modify one of the recipes listed below to change the number of tiles that will be imaged. For more information, see *Configuring Tile Selection* on page 213.

Single-Folder Paired-Read Method	Two-Folder Paired-Read Method
GA2-PEM_2x36_PE_v<#>.xml	GA2_FirstBase_<v#>.xml

3. Open the Illumina Genome Analyzer Data Collection software and select **File | Open Recipe**.
4. Depending on the method you are using, open one of the following recipes:

Single-Folder Paired-Read Method	Two-Folder Paired-Read Method
GA2-PEM_2x36_PE_v<#>.xml	GA2_FirstBase_<v#>.xml

5. Click **OK**.

   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. First Base recipes like wash and prime recipes are a special service recipe type. The log can be found in a date stamped run folder in the service directory of the data collection software folder. This directory should be cleared every 2–3 months.

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 20 minutes. At the end, a message indicates that the first-base incorporation chemistry is complete.

   The next step is to apply Scan Mix, and then determine the focal plane of the flow cell. This enables the software to automatically adjust 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 Mix* on page 123.

Loading the Flow Cell with Scan Mix

It is critical to introduce Scan Mix 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 Mix:

   Command: Pump
   To: Flowcell
   Solution: 3
   Volume: 100
   Aspiration Rate: 250
   Dispense Rate: 2500
3. With the cursor in the **Dispense Rate** field, press **Enter**.
4. Proceed to *Adjusting Focus* on page 124.

Adjusting Focus

The Genome Analyzer uses a third laser to maintain focus for each tile over the course of the run. In this section you will align the flow cell to the camera and calibrate the system for best focus.

Default XYZ Coordinates

When you initialize the Genome Analyzer, the lens orients itself using limit switches inside the instrument. The default 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 X=0 should put the left edge of lane 1 in the center of the screen. Y=0 puts the objective at the forward most point of the flowcell. Z=0 should put the clusters in focus.

The instructions in this section explain how to manually focus the instrument 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 returns to the home position.

Manual Controls

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

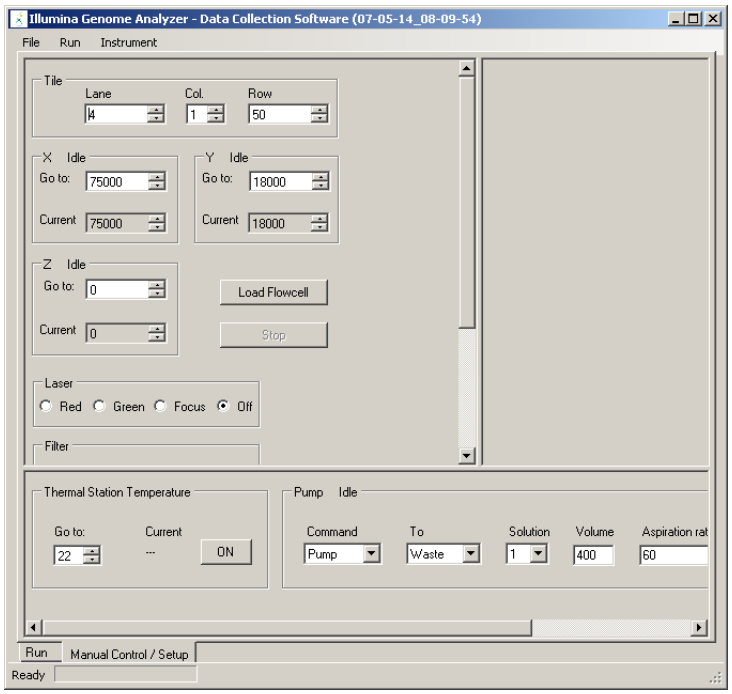


Figure 75 Manual Control/Setup Window

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

Table 16 Manual Controls

Area of the Screen	Description
Tile	Enter lane, column, and row coordinates to move the objective lens to a certain tile. The XY center point for flow cells is typically Lane 4, Column 1, Row 25. This is in the middle of the flow cell.
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** fields 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 reset Y=0 unless there is a significant hardware change, in which case an engineer will reset it. After you set the X value correctly, 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 and perform Autofocus Calibration before completing the run. Note: The focal position on the Z axis must be higher than Z= -20,000 (as noted above, it should ideally be zero), or the objective will not have enough room to move down without hitting the flow cell 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

First, you need to set the position of the flow cell along the X axis. The zero point should be at the left edge of Lane 1, near the bottom of the flow cell (Figure 76). This is done in five steps: *Moving the Stage*, *Setting Initial Focus*, *Finding the Left Edge*, *Confirming the Left Edge*, and *Setting Current X as Origin*.

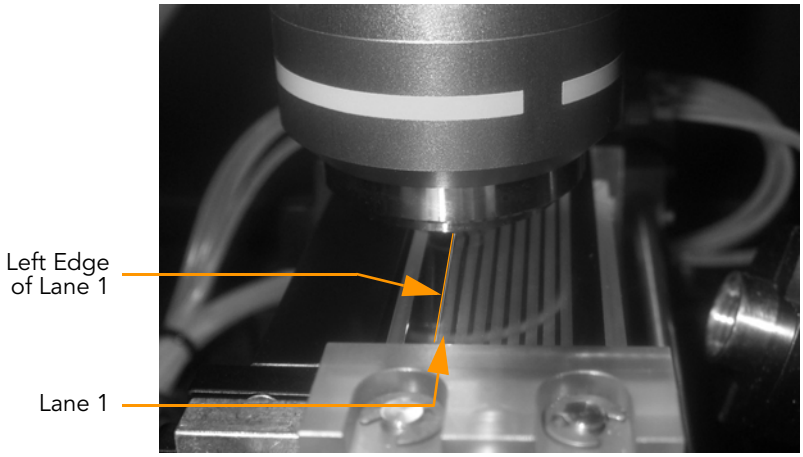


Figure 76 Left Edge of Lane 1

Moving the Stage

The sequence of stage adjustments described below is recommended to safely move the stage to the proper starting position, without running the risk of hardware collision.

1. Click the Manual Control/Setup tab.
2. Set the **Go To** values in the X and Y areas as follows:

   X (µm): 5000

   Y (µm): 15,000

   and press **Enter**.

   This moves the stage to a position near the middle of the flow cell.
3. Set **Z (nm)**: 0 (zero)

   and press **Enter**.

   This moves the objective stage close to the plane of focus prior to finding the edge of the flow cell. 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 133.
4. Set the **Go To** values in the X and Y areas as follows:

   X (µm): 0 (zero)

   Y (µm): 0 (zero)

   This moves the objective stage to a position near the bottom left of the flow cell. The left edge of Lane 1 is underneath the objective.

Setting Initial Focus

You need to set the initial focus in channel T, so you can see the left edge of Lane 1 as a sharp line. In a later stage, you will fine-tune the focus for all channels (*Adjusting the Z Axis*).

The Genome Analyzer images a 760 x 720 µm tile. In order to improve the ability to achieve focus and see images such as the ones below, use the zoom tool to view a square of approximately 1/10 of the whole tile image.

1. Set the following values:

   Laser: Green

   Filter: T

   Exposure (msec): 100

2. Right-click over the image and select **Auto Scale | On.**

If the clusters in the image are too dim, increase the exposure time to 200 msec. If the clusters are saturated (mouse over cluster and see if intensity reads 4095), reduce the exposure time.

3. Click **Take Picture**.
4. Use the descriptions here to decide in what direction to move the Z-axis.

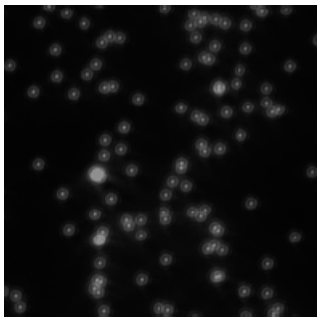


Figure 77 Lens Too High

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.

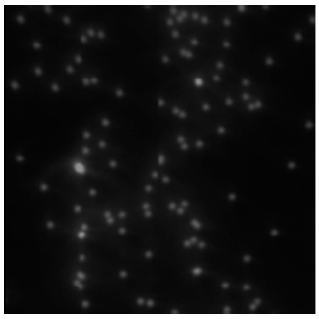


Figure 78 Lens Too Low

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.

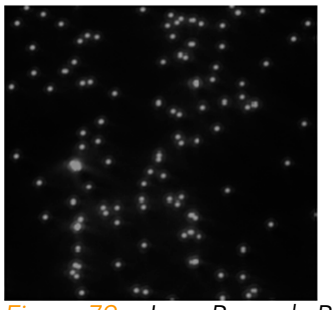


Figure 79 Lens Properly Positioned

Generally, start with a move no larger than 1000 nanometers.

NOTE

If you see you are very far away from focus, you may use 2500 nm moves; make sure not to step over the focus.

Initial moves should use the full tile image until the clusters become distinct points on the image. Subsequent moves can be smaller as you approach the optimal focus position. Zoom in on the image for final adjustment.

NOTE

The left part of the image may be dark without any clusters showing. This is to be expected, as this part lies outside of lane 1 where no clusters have formed.

5. Each time you move the Z position, take a new photo and check it. Once the clusters come into focal range, the Z movements should be in the range of 100–500 nm steps for each change.

NOTE

Problems with focusing may be caused by too much oil. Check whether the flow cell surface is dirty with oil; if so, you need to remove and clean the prism and flow cell. Follow the procedures described in *Cleaning and Installing the Prism* on page 110; skip *Performing First-Base Incorporation* on page 121 after reseating the prism and flow cell. Remember to add fresh Scan Mix prior to taking pictures.

6. Evaluate the image to determine whether first-base incorporation was successful. You should see many clusters.
7. 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 155).
   - If first-base incorporation was successful, continue with *Finding the Left Edge*.

Finding the Left Edge

Now you are ready to set the position of the flow cell along the X axis.

1. Click **Take Picture**. To see the left edge, zoom out to the whole tile image.

   The edge of Lane 1 appears on the screen. The screen is 2048 x 2048 pixels in size, with each pixel representing approximately 371 nm. The crosshair indicates the center of the image at (1024, 1024) pixels.

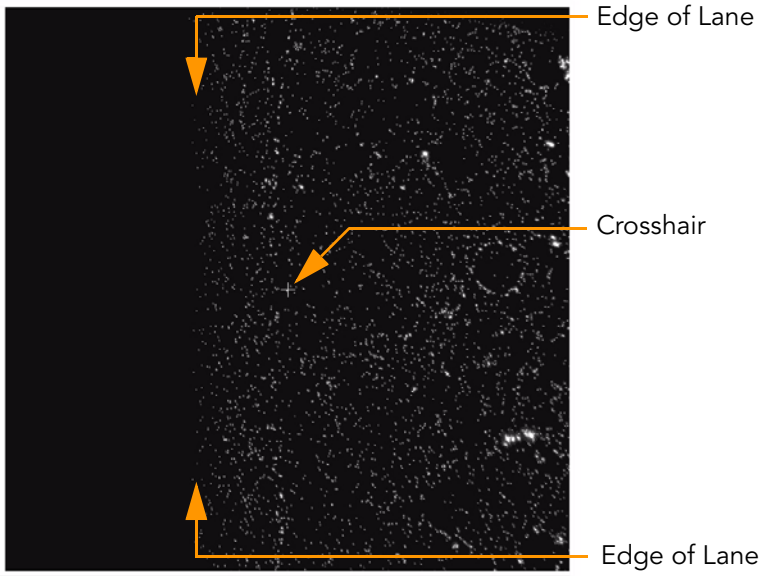


Figure 80 Crosshair at Center of Image

The left edge of Lane 1 should be close to the center of the image. To identify the distance between the edge and the crosshair, use your mouse to position the arrow over the edge of Lane 1. The pop-up window reveals the position of the arrow in pixels.

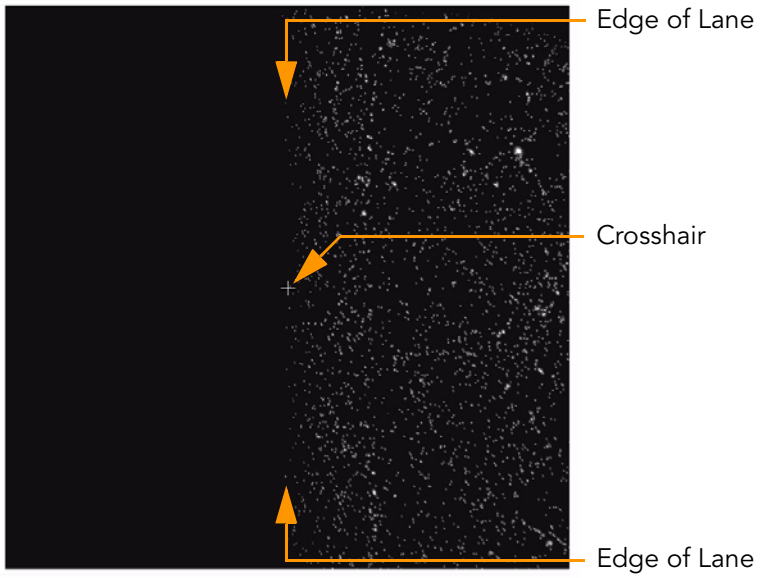


Figure 81 Left Edge of Lane 1 on the Screen

2. If the edge is more than 3–5 pixels 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 Lane 1 to the left; decrease it to move the edge to the right.

NOTE

You cannot change the X origin by more than 1000 µm at a time.

Confirming the Left Edge

Next, you need to confirm that you have found the left edge of Lane 1.

1. Set the following values:

   Laser: Green

   Filter: None

   Exposure (msec): 3

2. Click **Take Picture**.

   The edge of Lane 1 should appear on the screen as a blurred, wide line (Figure 82). The vertical crosshair should still be within 3-5 pixels of the blurred edge.

NOTE

If you do not see a blurred edge, or if the blurred edge is not in close proximity to the crosshair, you may be imaging the laser footprint edge. This indicates a misalignment in the instrument, and needs to be corrected by Illumina Field Service (for contact information, see *Technical Assistance* on page 4).

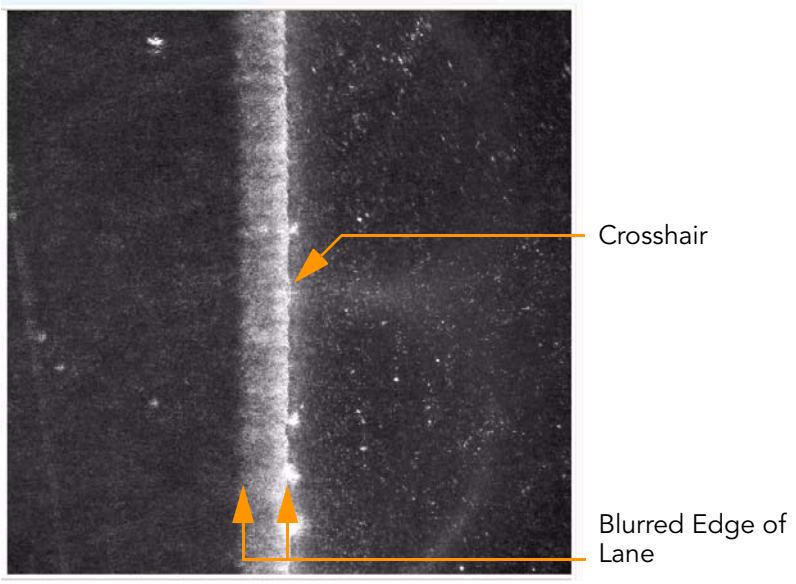


Figure 82 Blurred Edge of Lane 1

Do not skip the steps of aligning with a focused T image first, because unless the Z is focused first, alignment is meaningless.

Setting Current X as Origin

Now you are ready to set the origin.

1. Select **Instrument | Set Coordinate System | Set Current X as Origin**.
2. Click **OK** to confirm that you want to reset the coordinates and XY drift.

   This automatically adjusts the values in the HCMConfig.xml file. You will adjust the XY drift (or rotation of the part about the Z axis) in *Setting XY Drift* on page 131.

Adjusting the Y Axis

Do not adjust the Y axis.

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

Setting XY Drift

If the flow cell is rotated about the Z axis, the lanes will not be aligned with X at both ends of the lanes, and imaging may drift too far outside the lanes. To prevent this, you need to set a drift correction factor to adjust the X coordinate for each tile location.

Move the stage up in the Y axis to (0,35000) to find the left edge of lane 1 at the outlet end of the lane, and set the drift as described below. The edge at Y=35,000 is located the same way as the edge at Y=0 was found.

1. Set the **Go To** values in the X and Y areas as follows:

   X (µm): 5000

   Y (µm): 15,000

   and press **Enter**.

   This moves the stage to a position near the middle of the flow cell.
2. Set **Z (nm)**: 0 (zero)

   and press **Enter**.

   This moves the objective stage close to the plane of focus prior to finding the edge of the flow cell. 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 133.
3. Set the **Go To** values in the X and Y areas as follows:

   X (µm): 0 (zero)

   Y (µm): 35,000

   and press **Enter**.

   This moves the stage to a position near the upper left of the flow cell. The left edge of Lane 1 is underneath the objective.

CAUTION

The sequence of stage adjustments described above is recommended to avoid any chance of hardware collision.

4. Set the following values:

   Laser: Green

   Filter: T

   Exposure (msec): 100 (increase to 200 msec if the image is too dim)

5. Right-click over the image and select **Auto Scale | On.**

6. Click **Take Picture**.

   The edge of Lane 1 appears on the screen. The screen is 2048 x 2048 pixels in size, with each pixel representing approximately 371 nm. The crosshair indicates the center of the image at (1024, 1024) pixels. You may need to adjust focus for the new tile location (see *Setting Initial Focus* on page 127), since focus between tiles at opposite ends of the flow cells may differ.

   The left edge of Lane 1 should appear close to the center of the image. To identify the distance between the edge and the crosshair, use your mouse to position the arrow over the edge of Lane 1. The pop-up window will reveal the position of the arrow in pixels.

NOTE

For visual cues, see Figure 80 and Figure 81 in *Adjusting the X Axis* on page 126.

7. If the edge is more than 3–5 pixels 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 Lane 1 to the left; decrease it to move the edge to the right.)

8. Select **Instrument | Set Coordinate System | Set Current X as top-left edge to determine XY drift**.

9. The current Y is validated and the XY drift is computed. If the current Y and the computed XY drift are within range, the operator is prompted with a message box informing that "The drift was set to 0.nnnnn." If one of the values is out of range, perform the following:

   - If the Y coordinate is not large enough an error message is displayed to the operator, informing "Current Y=nnnnn is not far enough (min = mmmmm) from the coordinate system origin." Go back to step 1 and make sure you enter 35000 (thirty-five thousand) as Y value.
   - If the drift is too large, the following error message is displayed: "Flowcell XY Drift, resulting from TopLeftX = 333 and TopLeftY = 33333, exceeds allowed limit = 0.zzzz." The flow cell has not been registered correctly; check whether it is seated correctly against the pins. If necessary, clean and reload the prism and flow cell as described in *Cleaning the Prism* on page 111 and subsequent sections.

Confirming the Footprint

In this section you will confirm that the footprint is properly aligned.

1. Set the following values:

   Laser: Green

   Filter: T

   Exposure (msec): 100 (increase to 200 msec if the image is too dim, decrease if the image is saturated)

2. Right-click over the image and select **Auto Scale | On.**
3. Go to lane 4, column 1, row 25. Take an image with the T filter. Adjust focus if necessary.

   The left side of the image should have a black band with no clusters; this is the edge of the wall of the lane and will be cropped by the ROI. There should not be a black band on the top, bottom, or right of the column 1 image; if there is, the footprint is not aligned properly.
4. Next, move to column 2 (same lane and row) and take an image.

   Now, the black band without clusters should be on the right; no bands should be on the top, bottom, or left; if there are, the footprint is out of alignment.

If the footprint is misaligned, contact Illumina Field Service (for contact information, see Technical Assistance on page 5).

Adjusting the Z Axis

In this section, you take photos over Lane 4 to determine the optimal position of focus for the flow cell using the Focus Quality (FQ) metric. You should adjust the X axis and the XY drift before performing this procedure.

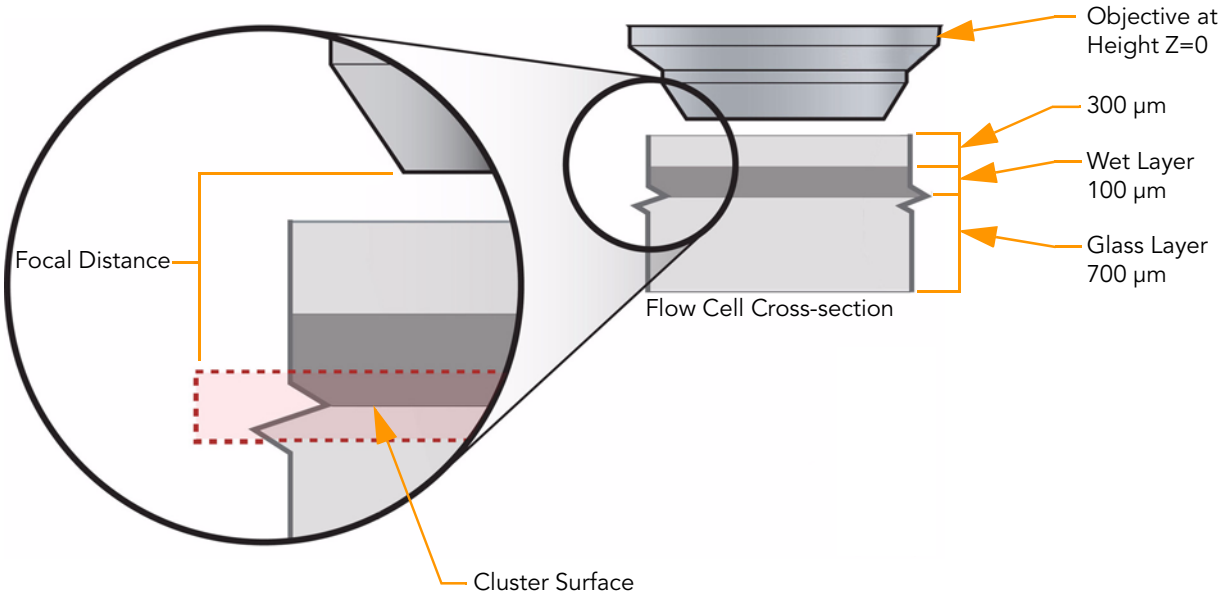


Figure 83 Focusing Z-Axis

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 25. This is the approximate center of the flow cell.

Typically, the center tile is used for setting the focus but you may prefer to move closer to the start of imaging at Lane 1, Column 1, Row 5.

Ensure that the calibration curve is of good quality and the flow cell drift is less than 5,000 nm. This is calculated for you by the First Cycle report from Run Browser. See *First-Cycle Report* on page 168.

2. Find the best focus for channel A by recording the FQ values for a range of Z positions:
   a. Set the following values:
      Laser: Red
      Filter: A
      Exposure (msec): 100 (increase to 200 msec if the image is too dim, decrease if the image is saturated)
   b. Right-click over the image and select **Auto Scale | On**
   c. Click **Take Picture**.
   d. Mouse over the image to see the FQ value, and record the FQ value and Z position in the lab tracking worksheet.

Focus Quality has a dependence on cluster size and has a "focused" optimum value from 74–85.

   e. Move the Z-position in increments of 200 nm, decreasing to 100 nm as the focus improves (higher FQ value). Repeat steps c–d each time you move the Z position.
   f. When you are able to identify the peak FQ value, move the Z-stage to the associated Z-position. Select **Instrument | Set Coordinate System | Set Current Z as Origin**.

3. Find the optimal focus for channels A, C, G, and T by recording the FQ values for a range of Z positions, and determining the Z position with the highest combined FQ value.
   a. Record the FQ values for channel A, C, G, and T by repeating steps 2c–d
      - *Channel A: Laser Red, Filter A.*
      - *Channel C: Laser Red, Filter C.*
      - *Channel G: Laser Green, Filter G.*
      - *Channel T: Laser Green, Filter T.*

b. Move the Z stage 500 nm up and down in 100 nm steps. Take pictures at every step, and record the FQ values and associated Z positions for all channels.

c. Add up all four FQ values for every Z position in the last column of the lab tracking worksheet.

d. Determine the Z position with the highest sum of FQ values. This is the optimal Z-position.

At the correct depth of focus, the FQ values will remain stable with little change over a range of 500 nm. You should calibrate auto focus and set Z=0 in the center of this 500 nm range.

e. Move the Z-stage to the optimal Z-position, and select **Instrument | Set Coordinate System | Set Current Z as Origin**.

4. Proceed to *Checking Quality Metrics* on page 136.

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.

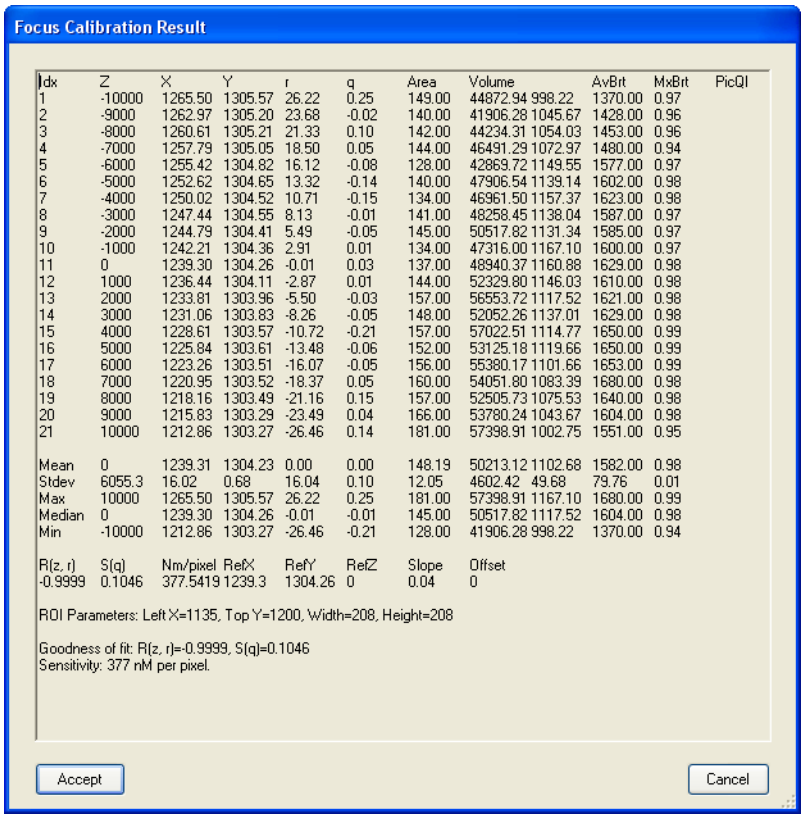


Figure 84 Autofocusing

6. Check the following specifications:

   Goodness of fit: ≥0.9900

   Absolute value of the sensitivity: 350–400.

   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 per *Adjusting the Z Axis* on page 133, 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 the Flow Cell with Scan Mix* on page 123 and continue from there.
   - If the calibration fails again, consult Illumina Technical Support.

   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 *Running a Report* on page 169; for an explanation of the laser spot metrics, see *Laser Spot Metrics: Measuring Autofocus Performance* on page 174.

8. Proceed to *Checking Quality Metrics in IPAR* on page 138.

Viewing Data in Run Browser

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

If you want to view the data in the Run Browser user interface, start Run Browser manually as described in *Checking First Cycle Results in the Flow Cell Window* on page 165. To view and analyze the data, and learn about Run Browser, follow the instructions in Chapter 5, *Run Browser Reports*.

After analyzing first-base incorporation data, proceed to *Completing Read 1* on page 139.

Checking Quality Metrics in IPAR

Integrated Primary Analysis and Reporting (IPAR) is a tool that displays key quality metrics in real time, so you can quickly decide whether or not to complete the run. To view and analyze the data, and learn about IPAR, see Chapter 6, *Integrated Primary Analysis and Reporting*.

Completing Read 1

If you are satisfied with the results of the first-base incorporation, follow these instructions to complete Read 1. A full sequencing run may take 48–72 hours.

Paired-end sequencing recipes are essentially identical to standard SBS recipes with the important exception that they end with a deblock cycle and the flow cell is then equilibrated in high salt buffer. This ensures any fluorescent background is removed prior to sequencing Read 2. Failure to use a paired-end recipe will result in a very high fluorescent background for Read 2 and may also compromise the intensity of the second read, both of which have a significant impact on data quality.

During a run, the operator can pause or stop the run by clicking **Stop**. The instrument is put into the "safe state." After pausing a run, the operator can resume it by clicking **Resume**. The protocol is resumed from the selected recipe item on the Recipe tab. If a run is stopped during imaging, 75 µl of Scan Mix must be pumped prior to resuming the run.

1. Depending on the method you are using, either resume the single-folder recipe or open the two-folder recipe:

Single-Folder Paired-Read Method	Two-Folder Paired-Read Method
GA2-PEM_2x36_PE_v<#>.xml	GA2_36Cycle_PE_v<#>.xml

   This step leaves the flow cell ready for Read 2 preparation by:
   - Ending with a deblock cycle
   - Flushing the flow cell with high salt buffer
2. Click **Start**.
3. When prompted, click **OK** to accept the name of the run folder. For more information about run folders, see Appendix A, *Run Folders*.
4. When the Autofocus Calibration dialog box appears, click **No** (you have already calibrated), and the Genome Analyzer resumes sequencing.

If the Genome Analyzer has been idle for several minutes, stay with the imaging of the first 10 tiles to confirm focus is good. If focus is off, redo manual focus on lane 4 as described in *Adjusting the Z Axis* on page 133, and click **Yes** when the Autofocus Calibration dialog box appears.

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

   If you are using the single-folder recipe, the following prompt appears when the run is complete: "Read 1 SBS Complete. Load Read 2 Prep Reagents onto the Paired End Module. Click OK to Start Read 2 Prep."

The flow cell can safely be left on the Genome Analyzer in High Salt Buffer for a period of three days after completion of Read 1 and before beginning Read 2 preparation.

Data Transfer for Paired-End Runs

If you are using the two-folder paired-read method, your data must be transferred to your network storage for data analysis after Read 1 is complete and before starting the recipe for Read 2.

Using the RoboCopy script to automatically copy files is recommended over manually copying files.

1. Check that all of the data from the Run Folder have been copied to your network storage location, including:
   a. Images
   b. Focus images (if stored)
   c. Log files
   d. Configuration files
   e. Calibration files
2. Confirm that all of the data has been transferred and checked.
3. Delete the Run Folder from the instrument data drive.

Do not attempt to start the Read 2 recipe until the deletion is complete. The disk space checking algorithm used by the instrument software may produce an error.

Preparing Reagents for Read 2 Preparation on the Paired-End Module

This protocol describes how to prepare reagents for Read 2 preparation on the Paired-End Module. The Paired-End Module is used to supply the Read 2 reagents to the Genome Analyzer via an external VICI valve.

All operations are performed on the Genome Analyzer.

- Primer Dehybridization
- Deprotection
- Resynthesis
- Linearization
- Blocking
- Primer Hybridization

Consumables

Illumina-Supplied

The following reagents and consumables are supplied with the Paired-End Read 2 Cluster Generation Kit (boxes 2 and 4):

- 0.1 N NaOH
- TE Buffer
- 5X Deprotection Buffer
- Deprotection Enzyme
- Cluster Buffer
- 10 mM dNTPs
- *Bst* DNA Polymerase
- Formamide
- 10X Linearization 2 Buffer
- Linearization 2 Enzyme
- BSA
- Ultra Pure Water
- Blocking Enzyme A
- 10X Blocking Buffer
- 2.5 mM ddNTP
- Blocking Enzyme B
- Wash Buffer
- Hybridization Buffer
- Rd 2 PE Seq Primer

User-Supplied

- 5 M Betaine solution
- 250 ml MilliQ water (for washing the Paired-End Module)

NOTE

All solutions for the Paired-End Module must be placed in 15 ml Polypropylene Falcon tubes.

Procedure

Reagent #21: Wash Buffer

1. Transfer 10 ml of wash buffer into a 15 ml Falcon tube.
2. Label the tube "Reagent #21."

Reagent #19: 0.1 N NaOH

1. Transfer 4 ml of 0.1 N NaOH into a 15 ml Falcon tube.
2. Label the tube "Reagent #19."

Reagent #20: TE Buffer

1. Transfer the TE solution into a 15 ml Falcon tube.
2. Label the tube "Reagent #20."

Reagent #9: Deprotection Premix

1. Prepare the following solution in a 15 ml Falcon tube:
   - Ultra Pure Water (1600 µl)
   - 5X Deprotection Buffer (400 µl)

   The total volume should be 2000 µl.
2. Mix by pipetting up and down using a 1 ml tip.
3. Label the tube "Reagent #9."
4. Place the tube on ice until you are ready to load it onto the Paired-End Module.

Reagent #10: Deprotection Mix

1. Prepare the following solution in a 15 ml Falcon tube:
   - Ultra Pure Water (1560 µl)
   - 5X Deprotection Buffer (400 µl)
2. Mix thoroughly by pipetting up and down using a 1 ml tip.
3. Add Deprotection Enzyme (40 µl).

   The total volume should be 2000 µl.
4. Mix by pipetting up and down using a 1 ml tip.
5. Label the tube "Reagent #10" and place on ice until you are ready to load it onto the Paired-End Module.

Reagent #15: Formamide

1. Transfer 8 ml of Formamide into a 15 ml Falcon tube.
2. Label the tube "Reagent #15."

Reagent #14: Cluster Premix

1. Ensure that the Cluster Buffer is completely thawed before use. Vortex briefly if necessary.
2. Prepare the Cluster Premix in a 50 ml tube as follows:
   - Ultra Pure Water (15 ml)
   - Cluster Buffer (3 ml)
   - 5M Betaine (12 ml)

   The total volume should be 30 ml.
3. Mix by gently inverting the tube five times.
4. Filter the Cluster Premix using a 0.2 µm cellulose acetate syringe filter and a 30 ml syringe.
5. Transfer 10 ml of the filtered Cluster Premix into a 15 ml Falcon tube.
6. Label the tube "Reagent #14."

Save the remaining Cluster Premix to prepare the *Bst* Mix.

Reagent #13: *Bst* Mix

1. Prepare the following solution in a 15 ml Falcon tube:
   - Filtered Cluster Premix (10 ml)
   - 10 mM dNTP Mix (200 µl)
   - *Bst* DNA Polymerase (100 µl)

   The total volume should be 10.3 ml.
2. Mix by slowly pipetting up and down using a 10 ml pipette.
3. Label the tube "Reagent #13."
4. Place the tube on ice until you are ready to load it onto the Paired-End Module.

Reagent #17: Linearization 2 Buffer

1. Dilute the 10X Linearization 2 Buffer to a 1X concentration with Ultra Pure Water as follows:
   - Ultra Pure Water (1800 µl)
   - 10X Linearization 2 Buffer (200 µ)

   The total volume should be 2000 µl.
2. Mix by pipetting up and down using a 1 ml tip.

3. Label the tube "Reagent #17."

Reagent #11: Linearization 2 Mix

1. Mix the following on ice at all times into a 15 ml Falcon tube:
   - Ultra Pure Water (1680 µl)
   - 10X Linearization 2 Buffer (200 µl)
   - BSA (20 µl)
2. Mix thoroughly by pipetting up and down using a 1 ml tip. Do not vortex.
3. Add Linearization 2 Enzyme (100 µl).
   The total volume should be 2000 µl.
4. Mix thoroughly, but gently, by pipetting up and down using a 1 ml tip.
5. Label the tube "Reagent #11."
6. Keep on ice until you are ready to load it onto the Paired-End Module.

Reagent #18: 1X Blocking Buffer

1. Dilute the 10X Blocking Buffer to a 1X concentration in a 15 ml Falcon tube as follows:
   - Ultra Pure Water (4500 µl)
   - 10X Blocking Buffer (500 µl)

   The total volume should be 5000 µl.
2. Mix by pipetting up and down using a 5 ml pipette.
3. Label the tube "Reagent #18."

1820 µl of 1X Blocking Buffer will be used to prepare Reagent #12 Blocking Mix.

Reagent #12: Blocking Mix

1. Prepare the following reagents in a 15 ml Falcon tube and mix on ice at all times:
   - 1X Blocking Buffer (1820 µl)
     (Obtained from Reagent #18 tube)
   - 2.5 mM ddNTP (80 µl)
   - Blocking Enzyme A (24 µl)
   - Blocking Enzyme B (100 µl)

   The total volume should be 2024 µl.
2. Mix by pipetting up and down using a 1 ml tip.
3. Label the tube "Reagent #12."
4. Keep on ice until you are ready to load it onto the Paired-End Module.

Reagent #16: Read 2 Sequencing Primer Mix

1. Prepare the following solution in a 15 ml Falcon tube:
   - Hybridization Buffer (1492.5 µl)
   - Rd 2 PE Seq Primer (7.5 µl)

   The total volume should be 1500 µl
2. Mix by pipetting up and down using a 1 ml tip.
3. Label the tube "Reagent #16."

Reagent Positions on the Paired-End Module

CAUTION

When you prepare and load these reagents onto the Paired-End Module and the Genome Analyzer, you must use them the same day.

Exception: The High Salt Buffer does ***not*** need to be made fresh for each sequencing run.

CAUTION

Ensure that all of the necessary software and configuration settings changes have been made by the FSE or FAS during the installation of the module prior to starting the first paired-end sequencing run.

The following figure illustrates the reagent positions on the Paired-End Module and the number associated with each position.

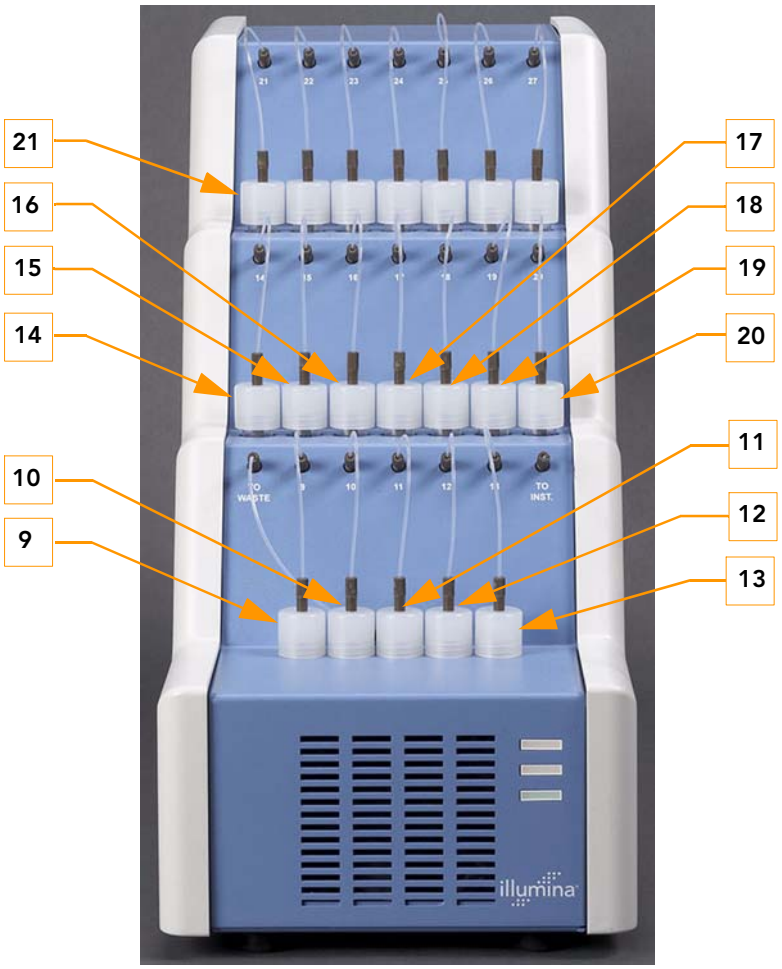


Figure 85 Reagent Positions on the Paired-End Module (Read 2)

Loading Reagents

The following table identifies the position of each reagent used in the Paired-End Module. Regardless of which recipe you use, the reagents always occupy the same positions.

Whenever you add a reagent to one of the Paired-End Module tubes, label it with the appropriate number as indicated.

It is essential to label all tubes correctly. Incorrect labeling can cause errors in the chemistry and damage to samples.

Table 17 Reagent Positions on the Paired-End Module and Read 2 Volumes

Position	Reagent	Initial Volume	Expected Volume After Priming	Expected Volume After Read 2
9	Deprotection Premix	2000 µl	1400 µl	800 µl
10	Deprotection Mix	2000 µl	1400 µl	800 µl
11	Linearization 2 Mix	2000 µl	1400 µl	800 µl
12	Blocking Mix	2024 µl	1424 µl	304 µl
13	*Bst* Mix	10300 µl	9700 µl	5380 µl
14	Cluster Premix	10000 µl	9250 µl	4690 µl
15	Formamide	8000 µl	7250 µl	3890 µl
16	Read 2 Sequencing Primer Mix	1500 µl	750 µl	150 µl
17	Linearization 2 Buffer	2000 µl	1250 µl	650 µl
18	1X Blocking Buffer	3180 µl	2430 µl	1830 µl
19	0.1 N NaOH	4000 µl	3250 µl	2050 µl
20	TE	3000 µl	2250 µl	1050 µl
21	Wash Buffer	10000 µl	9050 µl	3490 µl

Using the Paired-End Module

1. Leave the flow cell mounted on the Genome Analyzer.
2. The Paired-End Module should be fitted and installed to port position 8 of the Genome Analyzer internal VICI valve prior to starting Read 1 of the paired-end experiment.

Preparing for Read 2 on the Paired-End Module

The software guides you through the steps for the automated preparation for Read 2 on the Genome Analyzer with a Paired-End Module.

The Paired-End Module supplies all solutions to the flow cell. Temperature-sensitive reagents are located in cooled reservoirs on the Paired-End Module. After you finish preparing for Read 2, immediately begin Read 2 sequencing.

Prime the Paired-End Module

The priming steps are performed automatically using the internal priming pump on the Paired-End Module. The recipe primes each port position in turn and dispenses the waste to the waste bottle, bypassing the flow cell.

Single-Folder Paired-Read Method

1. Connect tubes 9–21 to the corresponding port position on the Paired-End Module.
2. Place the waste tube into the waste container.
3. Proceed to *Prepare for Read 2*. The single-folder recipe primes the Paired-End Module as part of the Read 2 preparation step in the protocol.

Two-Folder Paired-Read Method

1. Connect tubes 9–21 to the corresponding port position on the Paired-End Module.
2. Place the waste tube into the waste container.
3. Open the PEM_R2Prime_<v#>.xml recipe.
4. Click **Start**.

   The following prompt appears when priming is complete: "Priming complete. Press Enter or click OK to proceed to Read 2 preparation."

Prepare for Read 2

Preparation of Read 2 using the automated method takes approximately 4 hours and 20 minutes from the priming of the Paired-End Module. The process is fully automated and can be left to run unattended.

Single-Folder Paired-Read Method

1. Click **OK** to resume recipe GA2-PEM_2x36_PE_v<#>.xml.

   The recipe primes the lines and completes Read 2 preparation.

   The following prompt appears when Read 2 preparation is complete: "Read 2 Prep is complete. Load Read 2 SBS reagents onto the Genome Analyzer."

Two-Folder Paired-Read Method

1. Open the PEM_R2Prep_<v#>.xml recipe.
2. Click **Start**.

   The following prompt appears when Read 2 preparation is complete: "Flow cell rehybridized and ready for Read 2. Click OK to proceed."

Preparing Reagents for Read 2 on the Genome Analyzer

Follow these instructions to prepare reagents before loading them onto the Genome Analyzer. Required materials are provided in the 36-Cycle Sequencing Kit v2.

When you prepare and load reagents onto the Genome Analyzer, you must use them in a sequencing run the same day.

Unpack and Thaw Reagents

1. Remove the following reactive part components from -20°C storage and thaw them at room temperature or in a beaker containing deionized water. Do not microwave.
   - IMX36
   - FFN36
   - SMX36

   If you use the beaker method, make sure the water line does not reach the cap of the tube to prevent contamination.

   Leave the SDP36 in -20°C storage until you are ready to use it to make the Incorporation Mix.

It is important to keep the CMX away from the other components to avoid cross-contamination.

2. Remove the CMX36 from -20°C storage and thaw it at room temperature or in a ***separate*** beaker containing deionized water. Do not microwave. If you use the beaker method, make sure the water line does not reach the cap of the tube to prevent contamination.

After handling the CMX container, be sure to discard your gloves and replace them with a new pair each time.

3. Record the lot numbers of each reagent on the lab tracking worksheet.
4. Immediately after the reagents have thawed, place them on ice. Be sure to keep the CMX36 in a ***separate*** ice bucket during reagent preparation.
5. If the components from Box 1 are still frozen, thaw them in a container of deionized water.

Procedure

IMX36

Required Materials:

- FFN36
- IMX36
- SDP36

1. Transfer 1.75 ml of the FFN36 into the IMX36.
2. Remove the SDP36 tube from -20°C storage and briefly pulse centrifuge.
3. Transfer 220 µl of SDP36 to the IMX36 reagent (containing FFN36).
4. Cap the IMX36 (containing FFN36 and SDP36) tube tightly and invert five times to mix.
5. Centrifuge at 1,000 xg for 1 minute at 22°C.
6. Place the IMX36 on ice until you are ready to load it onto the Genome Analyzer.
7. Record the weight of the reagent in the lab tracking worksheet.

PR1

1. Invert the bottle of PR1 several times before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

PR2

1. Invert the bottle of PR2 several times to mix before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

PR3

1. Invert the bottle of PR3 several times before loading it onto the Genome Analyzer.
2. Record the lot number of the reagent on the lab tracking worksheet.
3. Record the weight of the reagent in the lab tracking worksheet.

SMX36

1. Invert the SMX36 tube several times to mix well, and then centrifuge at 1,000 xg for 1 minute at 22°C before loading it onto the Genome Analyzer.
2. Record the weight of the reagent in the lab tracking worksheet.
3. Place the SMX36 on ice until ready to load onto the Genome Analyzer.

CMX36

1. Invert the CMX36 tube several times to mix well, and then centrifuge at 1,000 xg for 1 minute at 22°C before loading it onto the Genome Analyzer.
2. Record the weight of the reagent in the lab tracking worksheet.

3. Place the CMX36 in a ***separate*** ice bucket until you are ready to load it onto the Genome Analyzer.
4. Discard your gloves and replace them with a new pair.

When you load the reagents onto the Genome Analyzer, load the CMX last to avoid cross-contamination.

Be sure to perform a pre-run wash before loading reagents onto the Genome Analyzer.

PW1

1. To prepare for the Genome Analyzer pre-run wash, aliquot 40 ml of PW1 into four 125 ml Nalgene bottles.
2. Aliquot 10 ml of PW1 into three 50 ml conical tubes.

See *Performing a Pre-Run Wash* on page 105 for pre-run wash instructions.

Sequencing Read 2

Do not turn off or re-initialize the Genome Analyzer as the X and Y stage coordinates will be lost, resulting in the inability to co-localize the two reads.

Do not make any changes to the map or configuration files between reads. This may result in the inability to co-localize the two reads.

The flow cell does not require remounting or cleaning and the leak test is not necessary.

Waste produced during Read 2 preparation on the Paired-End Module must be kept separate from waste produced during Read 2 sequencing on the Genome Analyzer. Waste from the Paired-End Module must be disposed of properly and in accordance with facility standards.

Single-Folder Paired-Read Method

1. Exchange the reagents used for Read 1 with fresh reservoirs from the reagents supplied in the SBS Sequencing Kit.
2. Replace all the reagent tubes on the Paired-End Module with Falcon tubes with at least 10 ml of MilliQ water.

Do not reprime reagents through the flow cell.

3. Click **OK** to resume the GA2-PEM_2x36Cycle_PE_v<#>.xml recipe and start first-base incorporation for Read 2.

   When the first-base incorporation is complete, the following dialog box appears: "Read 2 first base incorporation chemistry is complete. Press OK to continue. To perform manual focus and first base evaluation, press Cancel."

   "To use the existing calibrated focus, click OK to start imaging (the flow cell will automatically be flushed with Scan Mix). If you wish to refocus manually, click Cancel."
4. Click **OK** to accept the current calibrated focus and resume sequencing Read 2. The flow cell will automatically be flushed with Scan Mix (solution 3).

   If you wish to refocus manually, perform the following:

   a. Click **Cancel**.

b. To load the flow cell with Scan Mix, click the Manual Control/Setup tab.

c. In the Pump area, set the values as follows to pump Scan Mix:
Command: Pump
To: Flowcell
Solution: 3
Volume: 100
Aspiration Rate: 250
Dispense Rate: 2500

d. With the cursor in the **Dispense Rate** box, press **Enter**.

e. Perform manual focus and recalibrate the autofocus laser. See *Adjusting Focus* on page 124 for instructions.

f. Click **OK** to resume sequencing Read 2.

When imaging is complete, the following prompt appears: "Please evaluate the first base report data for Read 2. Click OK to proceed to first cycle imaging, or Cancel to stop."

5. Click OK to complete the sequencing of Read 2.

Two-Folder Paired-Read Method

1. Exchange the reagents used for Read 1 with fresh reservoirs from the reagents supplied in the SBS Sequencing Kit.

Do not reprime reagents through the flow cell.

2. Open the GA2_FirstBase_v<#>.xml recipe.

3. Click **OK** to run the recipe.

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.

When the first-base incorporation is complete, the following dialog box appears: "First base incorporation chemistry is complete. Press OK to continue. To perform manual focus and first base evaluation, press Cancel."

In the two-folder workflow, the focus calibration from the first read can be used for the second read. You must introduce Scan Mix, but not re-calibrate focus. This is possible only if the Genome Analyzer or the software has not been restarted in between the two reads.

4. Click **Cancel** to dismiss the Autofocus Calibration dialog box.

The next step is to apply Scan Mix, and then determine the focal plane of the flow cell. This enables the software to automatically adjust the focus during the run.

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

5. Load the flow cell with Scan Mix.
   a. Click the Manual Control/Setup tab.
   b. In the Pump area, set the values as follows to pump Scan Mix:
      Command: Pump
      To: Flowcell
      Solution: 3
      Volume: 100
      Aspiration Rate: 250
      Dispense Rate: 2500
   c. With the cursor in the **Dispense Rate** box, press **Enter**.
6. Perform manual focus and recalibrate the autofocus laser. See *Adjusting Focus* on page 124 for instructions. Reset only the Z axis as needed. Do not adjust the X axis or XY tilt.
7. Click **OK** to resume Read 2 sequencing.
8. Open the GA2_36Cycle_PE_v<#>.xml.
9. Click **OK** to complete the sequencing of Read 2.

Performing Post-Run Procedures

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

Weigh Reagents

Weighing reagents when a run is complete measures reagent consumption and fluidics performance.

1. Weigh all of the reagent bottles and record the results in the lab tracking worksheet.
2. 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

At the end of the second read, both the Paired-End Module and Genome Analyzer must be washed.

After completing Read 2, you must perform a thorough instrument wash. The wash flushes 4 ml of wash solution through each reagent port on the Genome Analyzer and 1 ml through each reagent port on the Paired-End Module. 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 the bottles and tubes containing PW1 that were used for the pre-run wash as follows:

   50 ml conical tubes with 10 ml of PW1 for port positions 1, 6, and 3

   125 ml bottles with 40 ml of PW1 for port positions 4, 5, and 7 (Position 2 already has a bottle containing PW1 attached).

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

2. Place at least 5 ml of MilliQ water in each Falcon tube in positions 9–21 on the Paired-End Module.
3. Bundle all waste tubes with parafilm, making sure to keep the ends even.
4. Place the bundled tube ends into a pre-weighed 50 ml conical tube.
5. Click the Run tab.
6. Select **File | Open Recipe**.
7. Open the GA2-PEM_PostWash_v<#>.xml recipe.

   This recipe washes the Genome Analyzer and the Paired-End Module.
8. Click **Start** and enter a file name.

   The wash cycle runs for approximately 60 minutes.

Using wash reagents other than the PW1solution in the Sequencing Kit, or failing to perform the wash cycle at the recommended intervals, may void the warranty.

Chapter 5

Run Browser Reports

Topics

Introduction

Run Browser is a utility that lets you assess the quality of run data on a workstation with access to the run folder on the server, without the need to 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 a graphical view of run metrics, so that you can decide whether or not to complete the run. By default, Run Browser automatically launches the First Cycle Report after the completion of first-base incorporation and the Quality Metric Deviation Report at the conclusion of a normal recipe.

Run Browser uses the following file:

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

User Interface

The Run Browser has five main windows:

- The **Flow Cell window** provides a graphical interface for quickly gathering data and seeing it in color with interactive tooltips.
- The **Report window** enables you to create textual reports on the same data.
- The **Metric Deviation Report window** summarizes significant cycle-to-cycle deviations of key QC values, so that problematic cycles in the run can be identified.
- The **ImageViewer** displays the image for a selected tile, if available.
- **Chart Windows,** which allow you to monitor run quality selected tiles.

Flow Cell Window

Launching Run Browser

Automatically

During installation, Run Browser is configured to automatically generate and open the first cycle report after first-base incorporation, and the Quality Metric Deviation Report at the end of a normal recipe. If you want to view the data in the Run Browser user interface, start Run Browser manually as described below.

Manually

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

- Navigate to c:\Illumina\SCS<version>\RunBrowser\bin\Release and double click RunBrowser.exe.

Illumina recommends that the Run Browser not be run on the instrument PC while data collection is in progress, and that it doesn't share the run folder on the instrument PC. Instead, Run Browser should be run on any other available workstation with access to the run folder on the server.

Open Log Files

1. Select **File | Open**.
2. Navigate from the workstation running Run Browser to the following location on the server with the run folder:
   - If you ran a First Base recipe:
     Illumina\SCS<version>\bin\Service\<DateTimeStamp>\
   - If you ran a Normal Recipe:
     Runs\<run folder>\
3. Open the log files from the recent run (Ctrl- or Shift-select to select more than one file).

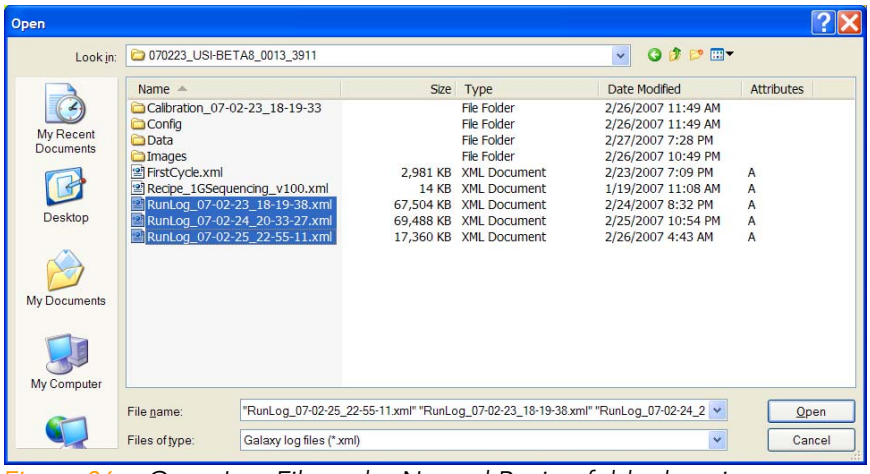


Figure 86 Open Log File at the Normal Recipe folder location

It may take a few moments for the tiles to load. A progress bar in the lower-left hand corner indicates log file loading progress and the number of files loaded. If IPAR-produced *.bro files exist in <$Run-

Folder>\data\runbrowser\, those will be loaded automatically for the cycles whose log files have been opened.

4. The data from the run appear in the Flow Cell window, along with a map of the flow cell. Select all tiles on the map of the flow cell by using the right button of your mouse.

   You can check data in this window by selecting different quality metrics and mousing over the flow cell to see values for each tile (see *Using the Flow Cell Window* on page 161).

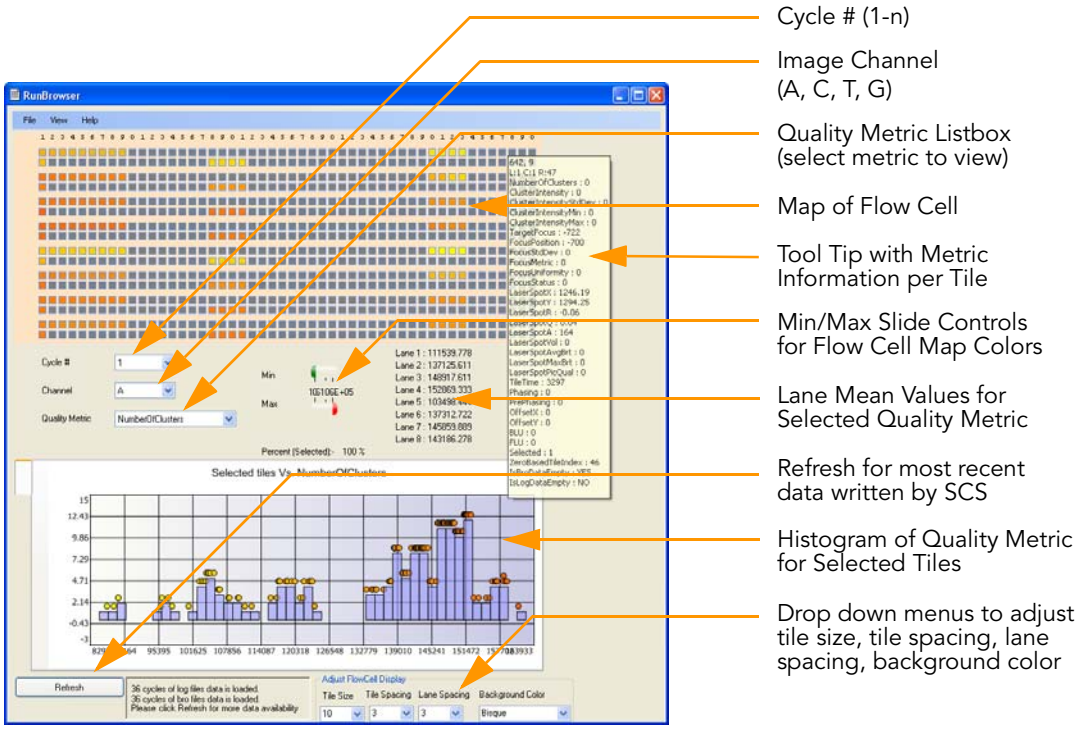


Figure 87 Flow Cell Window

Changing Automatic Launch Settings

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

1. Navigate to C:\Illumina\SCS<version>
2. Open RunConfig.xml in a text editor.
3. Edit the following parameters:

Setting	Description
`<RunBrowserExe>C:\Illumina\SCS<version>\RunBrowser\bin\Release\RunBrowser.exe</RunBrowserExe>`	Sets the path to the Run Browser executable.
`<EnableRunBrowserAutoLaunch>true</EnableRunBrowserAutoLaunch>`	Determines whether Run Browser launches automatically.

If you do not want Run Browser to display, export a report, or show the report automatically, do the following:

1. Navigate to <install directory>\Illumina\RunBrowser_v[#]\bin\Release
2. Open RunConfig.xml in a text editor.
3. Edit the following parameters:

Setting	Description
`<ShowRunBrowser>false</ShowRunBrowser>`	Does not open the Run Browser user interface automatically.
`<ExportReport>false</ExportReport>`	Does not export the Run Browser report automatically.
`<ShowReport>false</ShowReport>`	Does not open the Run Browser report automatically.

Using the Flow Cell Window

Refreshing Run Browser Data

Run Browser has a Refresh feature in the bottom-left corner of the Flow Cell Window (Figure 87). Run Browser is able to load a log file that is currently being written by the SCS software; clicking **Refresh** loads the most recent data. In addition, if any new log files are added, one click on **Refresh** loads those new files into Run Browser.

Browse Loaded Metric Data

Using the drop down menus named Cycle #, Channel, and Quality Metric (see Figure 87), you are able to navigate to the data of a certain cycle, certain channel and certain quality metric. Hovering the mouse over a tile on the flow cell map will show the data for a particular tile on the tool tip.

Change Flow Cell Display

You can adjust minimum and maximum values of quality metrics below which and beyond which color of tiles should be green and red respectively (Figure 87, Min/Max Slide Controls for Flow Cell Map). The color of those tiles that have metric values in between minimum and maximum are calculated and updated accordingly.

Dropdown lists at the bottom of the Run Browser window let you change tile size, tile spacing, lane spacing, and background color of the flow cell map.

ImageViewer

Once data are loaded into Run Browser, you can view images by double-clicking a tile on the flow cell map (Figure 88). ImageViewer displays the image if it exists for that tile for the selected cycle.

The image captured by the camera has a dynamic range of 12 bits per pixel (range of 0–4095), while the monitor displays a range of 8 bits (range of 0–255). The ImageViewer will autoscale the image that is chosen, setting the Maximum intensity value as 255 and the Minimum value as 0. You can customize the sensitivity of the image in the following ways:

- The sliders allow you to contrast portions of the image:
  - By reducing the Max slider, you can differentiate darker portions of the image (at the expense of saturating the higher intensity pixels).
  - By increasing the Min slider, you can differentiate brighter portions of the image (at the expense of losing the lower intensity pixels).

- Deselecting the GrayScale checkbox gives two more options:
  - The BGR color setting improves viewing of lower intensity contrasts.
  - The RGB color setting improves viewing of higher intensity contrasts.

In addition, ImageViewer displays two histograms that help you judge the images. The input histogram displays the raw intensity value of the pixels on the Y axis and the number of pixels in base 2 logarithmic scale on the X axis. The output histogram displays the 8-bit adjusted values. Note that the number of pixels will increase or decrease based upon the adjustment of the Min/Max sliders.

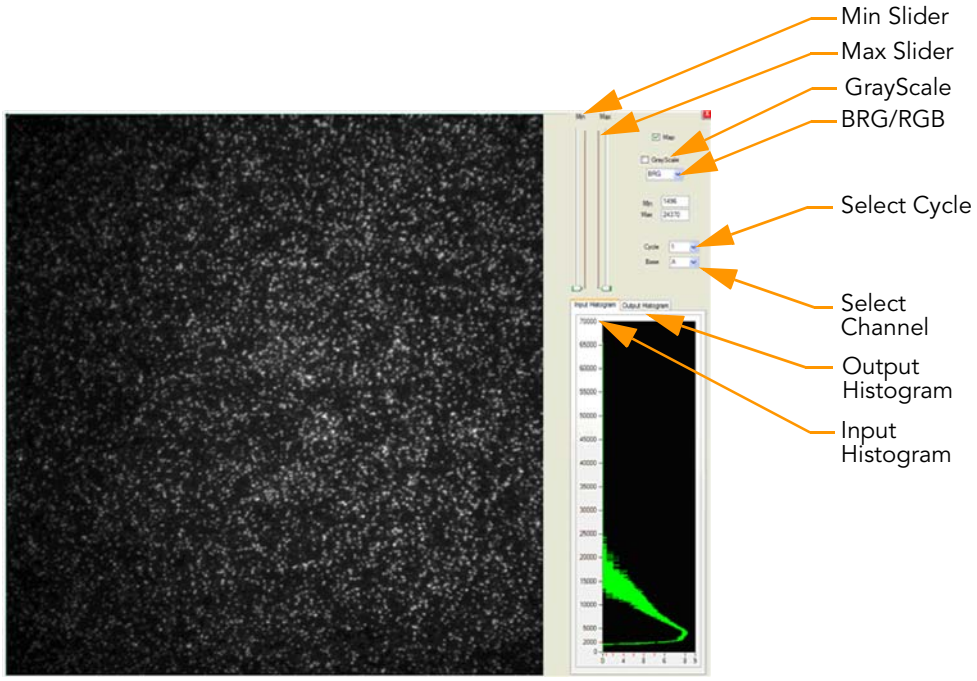


Figure 88 ImageViewer

Histogram of Selected Quality Metrics

You can display selected quality metrics for selected tiles in the histogram at the bottom of the Flow Cell window. Select the desired cycle, channel and quality metric, then click and drag the mouse to select a section of the flow cell in the flow cell map. Release the mouse button to plot a histogram of the selected quality metric for the selected tiles. Tiles appear as circles on the chart and have tool-tip information available.

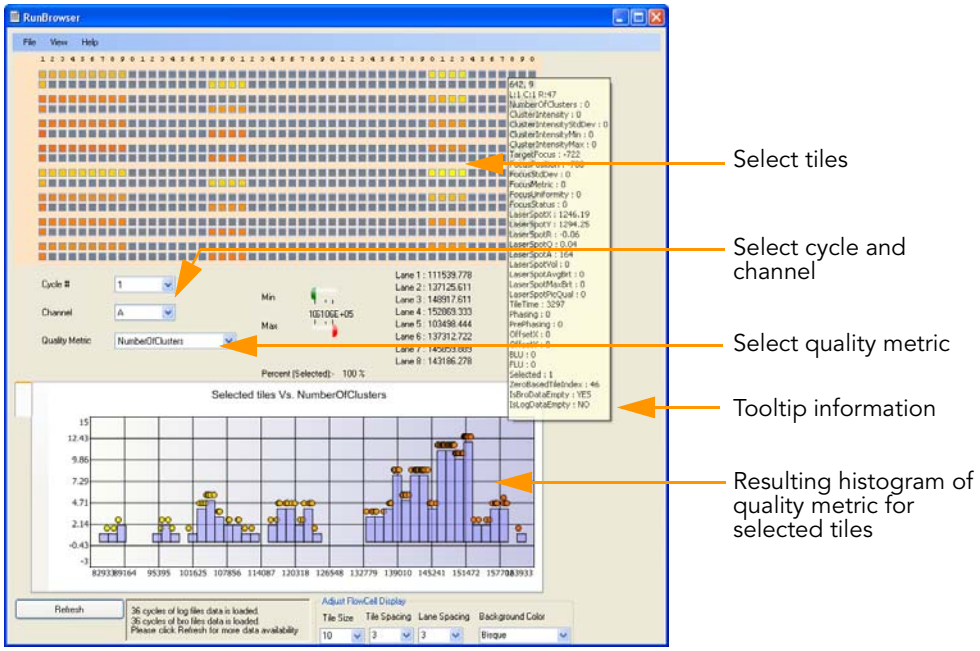


Figure 89 Histogram of Selected Quality Metrics.

Charts for Selected Tiles

You can generate two additional charts for selected tiles to monitor run quality. Click and drag the mouse to select a section of the flow cell in the flow cell map. Right click to view the context menu on the flow cell map, which lets you plot two types of graphs or copy the flow cell map graphic into the clipboard.

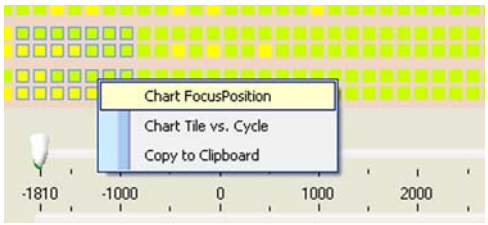


Figure 90 Select Tiles and Chart

Chart FocusPosition plots the focus position against selected tiles, for different cycles (Figure 91).

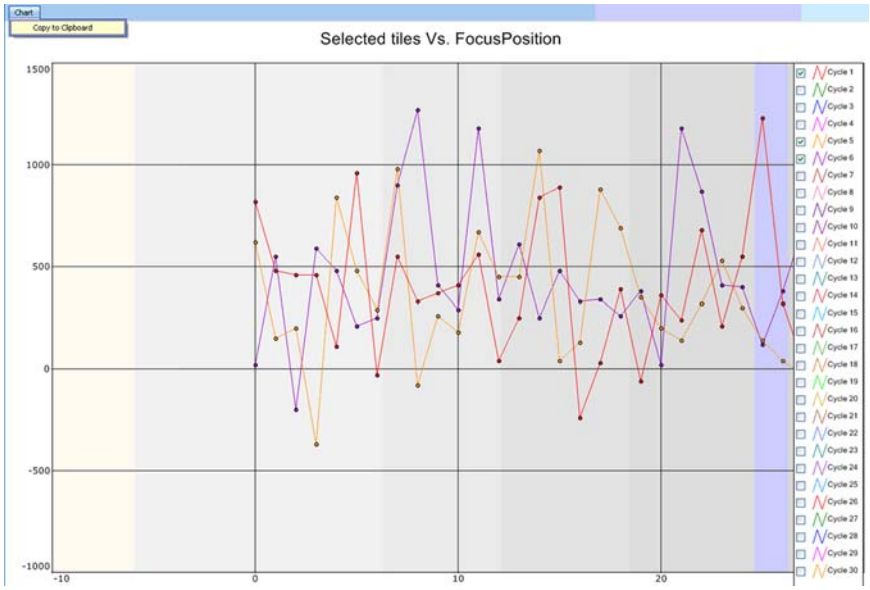


Figure 91 Chart FocusPosition

Chart Tile vs. Cycle plots the focus position against selected cycles, for different tiles (Figure 92).

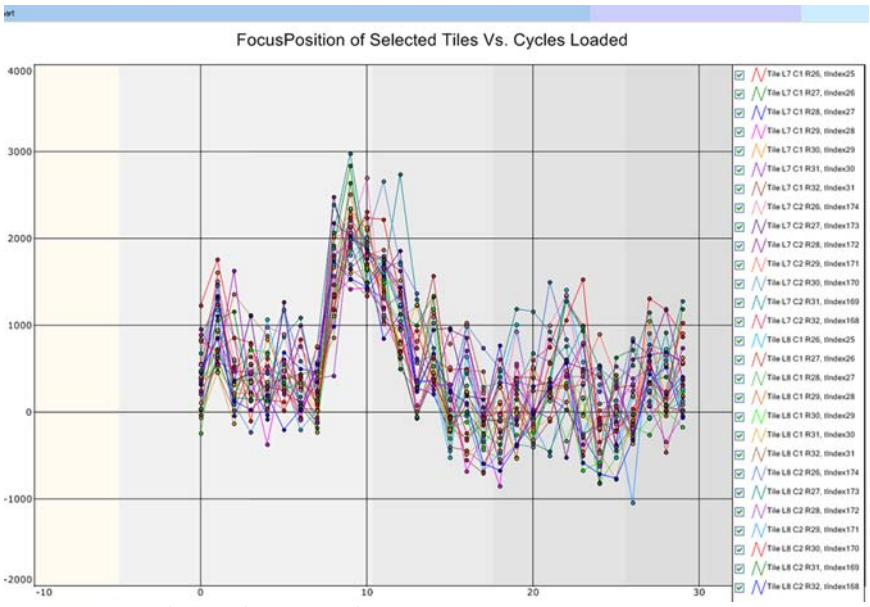


Figure 92 Chart Tile vs. Cycle

The charts have a Copy to Clipboard feature. This feature can be useful for copying graphics into a presentation.

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 (*Running a Report* on page 169) to see a complete summary of the data.

1. Follow the instructions in *Launching Run Browser* on page 159 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.

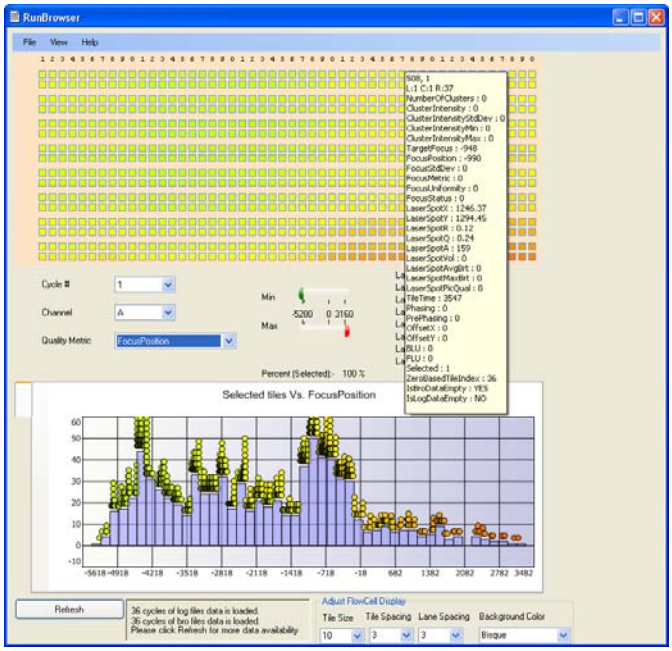


Figure 93 Focus Stage Level

3. To check the cluster intensity values:
   a. Select Cluster Intensity 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.

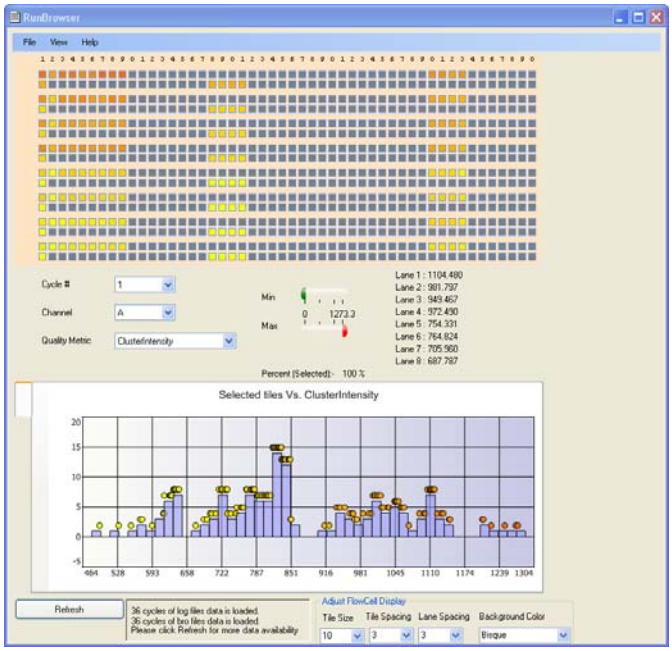


Figure 94 Cluster Intensity Levels

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 18 Cluster Intensity Values

	A	C	G	T
High Confidence	> 650	> 650	> 1000	> 1200
Reasonable Confidence	> 350	> 350	> 650	> 700
Low Confidence	< 250	< 250	< 350	< 400

4. To check the focal quality:

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.

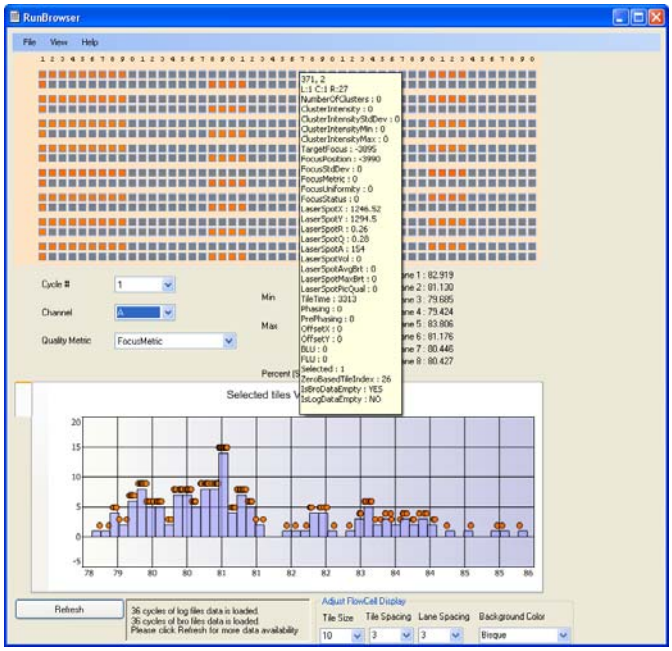


Figure 95 Run Browser Focus Metric

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 70.

If the focus metric for a lane is lower than 70, the flow cell surface may be dirty, or oil may not cover the entire flow cell-prism interface. Inspect the flow cell. You may need to clean and reload the prism and flow cell as described in *Cleaning and Installing the Prism* on page 110 and subsequent sections.

Report Window

Report Types

Run Browser provides two types of reports generated from the Report Window: 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.

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 *Cycle-to-Cycle Metrics: Measuring Quality Deviations* on page 177.

Running a Report

After opening the log file as described in *Launching Run Browser* on page 159, generate text reports that you can then print, save, or export as follows:

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

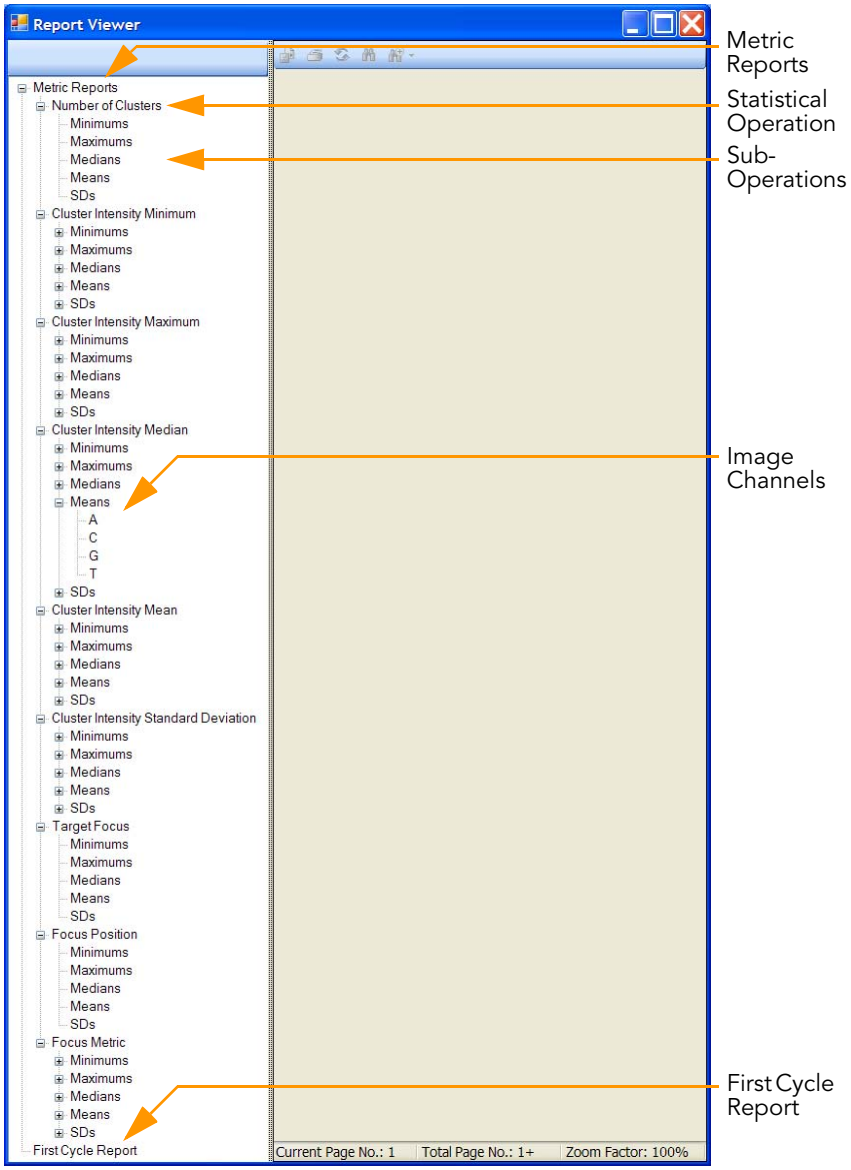


Figure 96 Empty Report Window

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

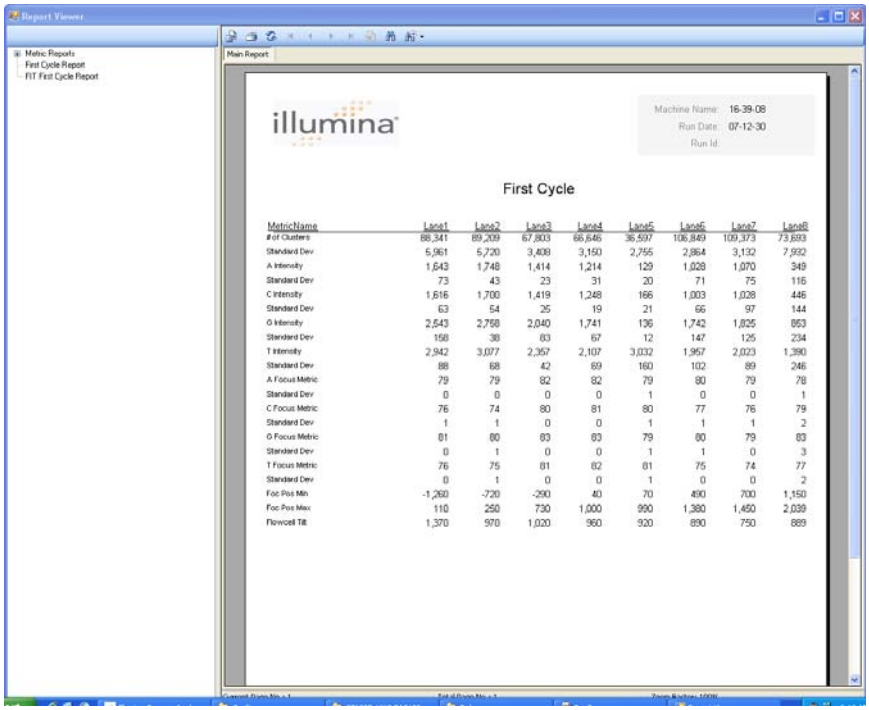


Figure 97 Sample First-Cycle Report

3. 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.

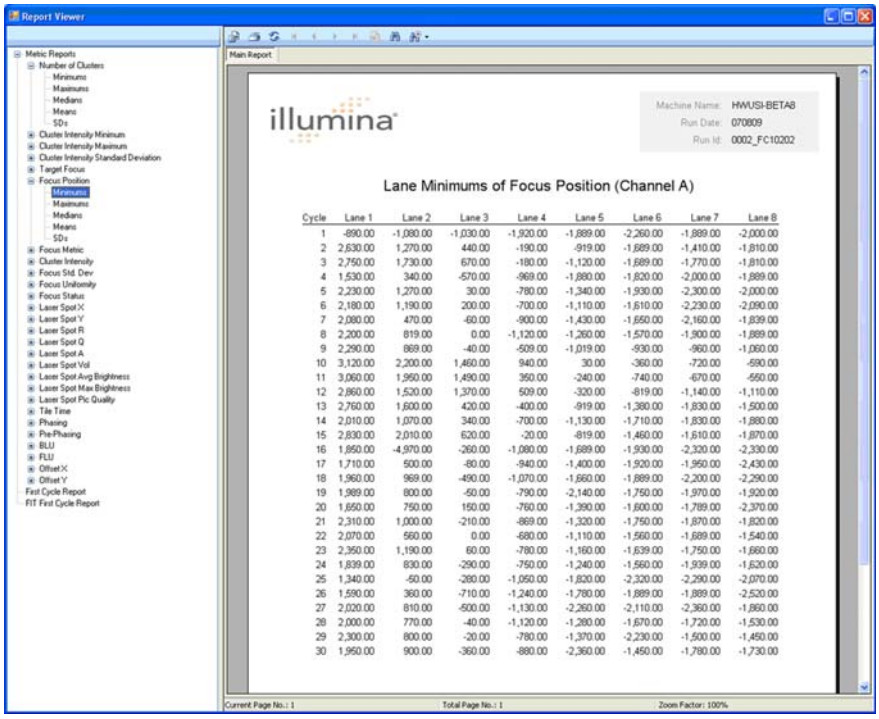


Figure 98 Sample Metric Report

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

Table 19 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
	Display the 1st page of the report
	Shows the previous page of the report
	Shows the next page of the report
	Shows the last page of the report

Table 19 Run Browser Report Viewer Buttons (Continued)

Button	Function
	Go to page (specify page in pop-up window)
	Find text
	Zoom view (options provided in cascading menu)

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. See Table 18 on page 166 for confidence levels for intensities.

Table 20 Measuring Cluster Quality

Statistical Operation	Description
Cluster Intensity Median	The median intensity of the selected channel, per tile.
Cluster Intensity Mean	The mean intensity of the selected channel, per tile.
Cluster Intensity Minimum	The minimum cluster intensity of the selected channel, per tile.
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.

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 IPAR records information on the Z-stage position and image quality of each tile.

Table 21 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.

Table 21 Measuring Image Quality (Continued)

Statistical Operation	Description
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 70.
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 22), 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 22 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 (The volume of the spot exceeds threshold).	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 subsystem over the course of the imaging cycle. They can indicate when there is air in the system. Histograms of these metrics will highlight problematic tiles. You should use these metric to look for outlying values from a mean.

Table 23 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.
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 the area.
Laser Spot Maximum Brightness (Max Brt)	The maximum gray value of the pixels in the laser spot, divided by the 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.

Phasing Metrics: Measuring Cycle Independence

The Genome Analyzer Pipeline uses phasing and prephasing to remove signal components from the next (prephasing) and previous (phasing) cycles. The window used in the Pipeline is cycles 3-12, from which the median per lane is calculated. IPAR mimics the Pipeline calculations for phasing and prephasing ONLY for cycle 12. This cycle can be viewed to determine the amount of interference from adjacent cycles.

To view these report, expand **Metric Reports** > **Phasing** > **Median** or **Metric Reports** > **Pre-Phasing** > **Median** in the Report Viewer.

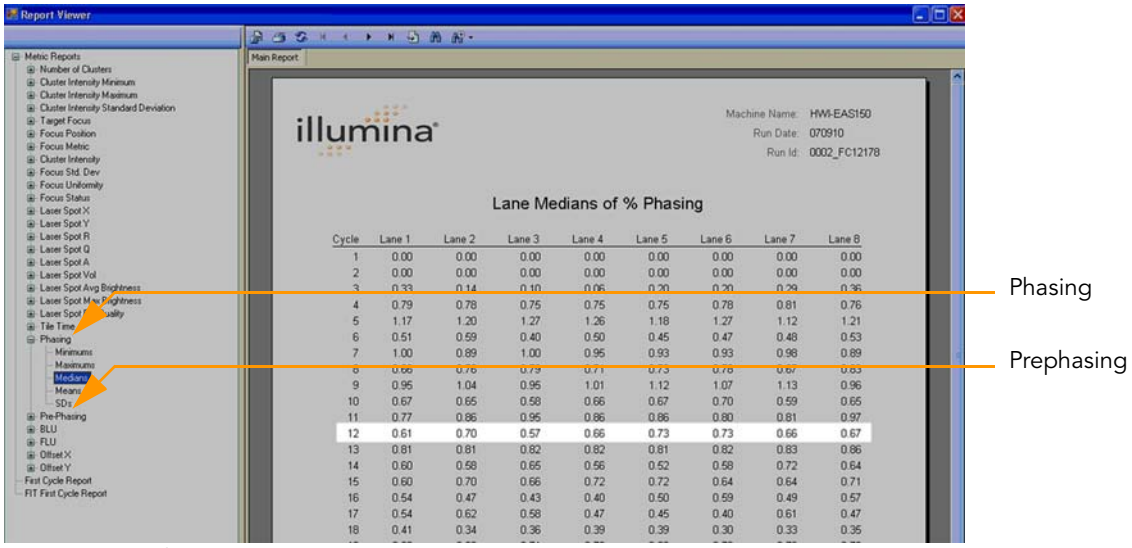


Figure 99 Phasing Report

Phasing/prephasing calculation requires intensity data from previous cycles. IPAR performs phasing/prephasing calculations after the last tile from the cycle is processed. The normal range of 0.1% to 1.0% is the signal contribution percentage from the next or previous cycle to the current cycle. High values (1.0%–2.0%) may indicate reagent or fluidics problems.

NOTE

While RunBrowser displays phasing and prephasing numbers starting from cycle 3, only phasing values from cycle 12 should be expected to match the offline analysis values. All phasing/prephasing values from other cycles are for research purposes only.

In addition, in order for phasing and prephasing values to match offline analysis values, the default offsets and matrix file selections must be comparable.

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, then the values will be within 5% of the numbers reported by offline analysis.

Tile Time: Measuring Software Overhead

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

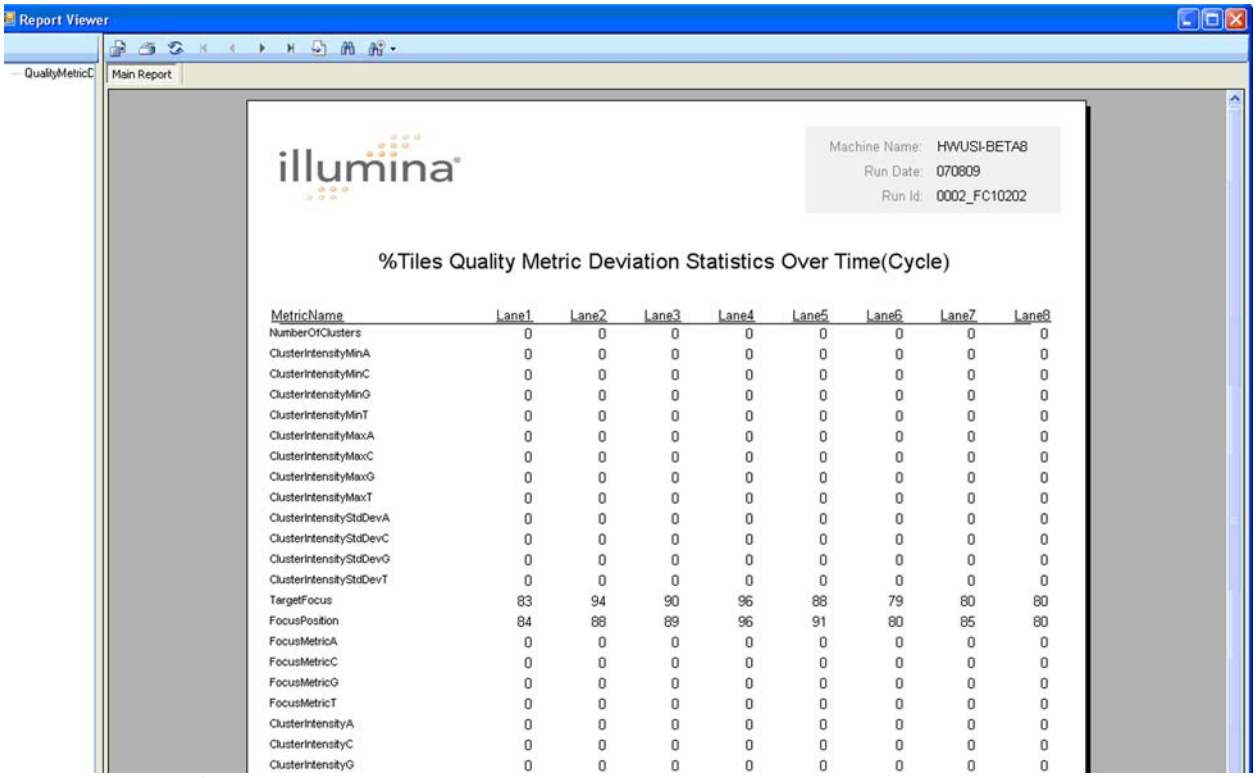
Metric Deviation Report Window

The Quality Metric Deviation (QMD) report summarizes significant cycle-to-cycle deviations of key QC values, so that problematic cycles in the run can be identified.

Running a Metric Deviation Report

After opening the log file (see *Launching Run Browser* on page 159), select **View | Metric Deviation Report** on the Run Browser main window to launch the QMD report, which may take several minutes.This report runs on the data of all cycles currently loaded into Run Browser and finds the percentage of tiles per lane that crosses a threshold of (approximately) 25% from adjacent (previous and next) cycles, and summarizes the result into a report.

For an explanation of the Run Browser Report Viewer Buttons, see Table 19 on page 171.



MetricName	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Lane7	Lane8
NumberOfClusters	0	0	0	0	0	0	0	0
ClusterIntensityMinA	0	0	0	0	0	0	0	0
ClusterIntensityMinC	0	0	0	0	0	0	0	0
ClusterIntensityMinG	0	0	0	0	0	0	0	0
ClusterIntensityMinT	0	0	0	0	0	0	0	0
ClusterIntensityMaxA	0	0	0	0	0	0	0	0
ClusterIntensityMaxC	0	0	0	0	0	0	0	0
ClusterIntensityMaxG	0	0	0	0	0	0	0	0
ClusterIntensityMaxT	0	0	0	0	0	0	0	0
ClusterIntensityStdDevA	0	0	0	0	0	0	0	0
ClusterIntensityStdDevC	0	0	0	0	0	0	0	0
ClusterIntensityStdDevG	0	0	0	0	0	0	0	0
ClusterIntensityStdDevT	0	0	0	0	0	0	0	0
TargetFocus	83	94	90	96	88	79	80	80
FocusPosition	84	88	89	96	91	80	85	80
FocusMetricA	0	0	0	0	0	0	0	0
FocusMetricC	0	0	0	0	0	0	0	0
FocusMetricG	0	0	0	0	0	0	0	0
FocusMetricT	0	0	0	0	0	0	0	0
ClusterIntensityA	0	0	0	0	0	0	0	0
ClusterIntensityC	0	0	0	0	0	0	0	0
ClusterIntensityG	0	0	0	0	0	0	0	0

Figure 100 Quality Metric Deviation Report

Cycle-to-Cycle Metrics: Measuring Quality Deviations

For QMD calculations, a three cycle window is analyzed to determine if there is a significant change in a quality score that is not part of a directional trend.

- Directional deviations are not counted. That is, tiles with progressively increasing trends or progressively decreasing trends across the moving window of 3 cycles will not be counted as deviations.
- Only ripples in trends are counted. For example if a tile's metric value increases by 25% from one cycle to the next cycle, and then decreases by more than 25% in the following cycle, that tile will be counted as a deviant for that metric. Similarly if a tile's metric value decreases by 25% from one cycle to the next cycle, and then increases by more than 25% in the following cycle, that tile will be counted as a deviant.
- Calculations are only performed for data loaded into Run Browser. If you only load one Run Log file, this report will only produce data for that one log file and any associated *.bro files.
- If a metric is missing for a particular cycle it is not counted.
- The percentage of tiles with a deviation is across all cycles loaded in Run Browser. If a tile shows deviation at multiple cycles, it is only counted once for the percentage calculations.
- The following formula is used to flag deviants (where V = quality Value, n = cycle number):

  To flag V shaped ripples in trends: $2(Vn) / (Vn-1 + Vn+1) < 0.75$

  To flag ^ shaped ripples in trends: $2 (Vn) / (Vn-1 + Vn+1) > 1.25$

Chapter 6

Integrated Primary Analysis and Reporting

Topics

Introduction

The Integrated Primary Analysis and Reporting system (IPAR) v1.0 brings up multiple valuable features to the GA software. These include online image analysis resulting in up to 50% reduction of the total analysis time for the data, minimizing the need to transfer large image files across the network, and provide real time feedback for the progress of the experiment. You can view the data for all tiles quickly in an overview window, and navigate into the data for specific lanes, tiles, or metrics.

The primary function for IPAR is to do image analysis. In addition, IPAR visualizes results using calculated intensities and quality parameters from image analysis. This enables you to quickly decide whether or not your run is progressing as expected. IPAR is configured to run automatically on a dedicated server, although the IPAR user interface, the Analysis Viewer, is integrated with the Genome Analyzer instrument control software.

Audience and Purpose

This guide is for laboratory personnel and other individuals responsible for operating IPAR with Sequencing Control Studio (SCS) v2.01 or later.

In addition to the instructions on using IPAR, this user guide contains a section on frequently asked questions that provides additional support.

User Interface

The Analysis Viewer has two main types of plots:

- The **overview display** provides a graphical interface integrated in the Genome Analyzer control software to quickly gather quality data and view it in color with interactive tooltips. Multiple quality metrics can be displayed in one plot. This allows you to easily find the areas that may need your attention.
- **Individual parameter plots** allow you to monitor the raw values of individual selected metrics. Only one type of quality metric can be monitored per plot. This lets you look in depth at the raw values for specific metrics, so you can compare them with established standards or previous runs.

Starting up IPAR

You need to be logged on to the IPAR computer before starting the IPAR analysis.

1. Make sure the UPS is powered up, then power on IPAR.

   Before proceeding to the next step, wait until the IPAR server has started up (usually 3 minutes).

2. Log on to the IPAR server:
   a. Open the remote desktop application on the instrument computer by selecting **START | All Programs | Accessories | Remote Desktop Connection** from the task bar.
   b. Connect to 192.168.137.20.
   c. Log on using the default values:
   Username: sbsuser
   Password: sbs123

Depending on your system configuration, you may also log on to the IPAR computer using the shared keyboard and monitor at the instrument computer, or the keyboard and monitor at the IPAR computer.

3. IPAR will start automatically as long as you have an active logon session on the IPAR computer. Each time a recipe is started or resumed the Genome Analyzer will automatically initiate a new IPAR session.

- There is no requirement to turn on the Genome Analyzer, instrument computer and IPAR computer in any given order.
- You do not need to reboot the IPAR computer between runs, unless you experience any connection problems when starting a run.

Using the Analysis Viewer

Introduction

The multi-tabbed visualization control Analysis Viewer displays data plots using a tile-based X axis, and a scaled or real Y value. Select the Analysis Viewer tab to bring up the Analysis Viewer panel (Figure 101).

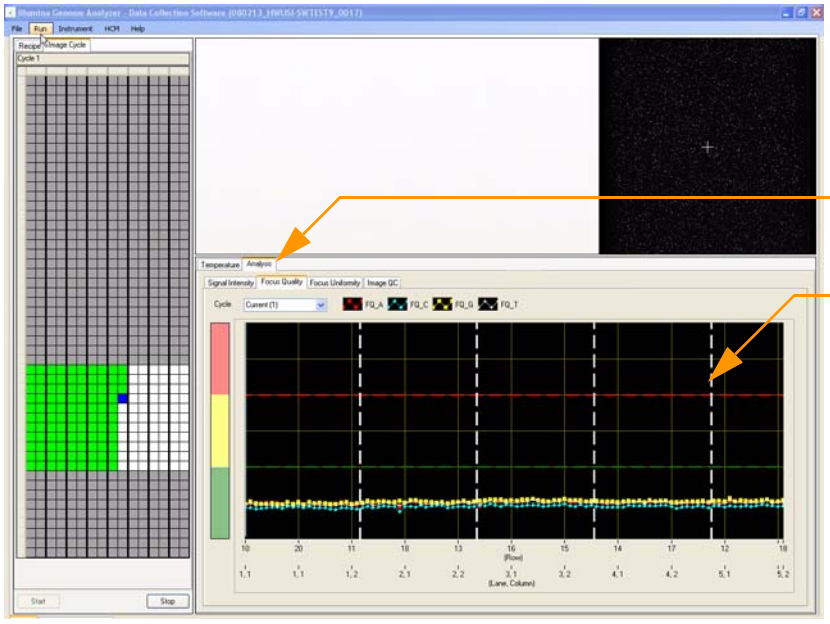


Figure 101 Integrated IPAR Analysis Viewer

Overview Display

The overview display allows you to monitor different quality metrics in the same plot (Figure 102). Different sets of quality metrics will be plotted by selecting a different quality metric tab. The meaning of these quality metrics is explained in *Quality Metrics in IPAR* on page 188.

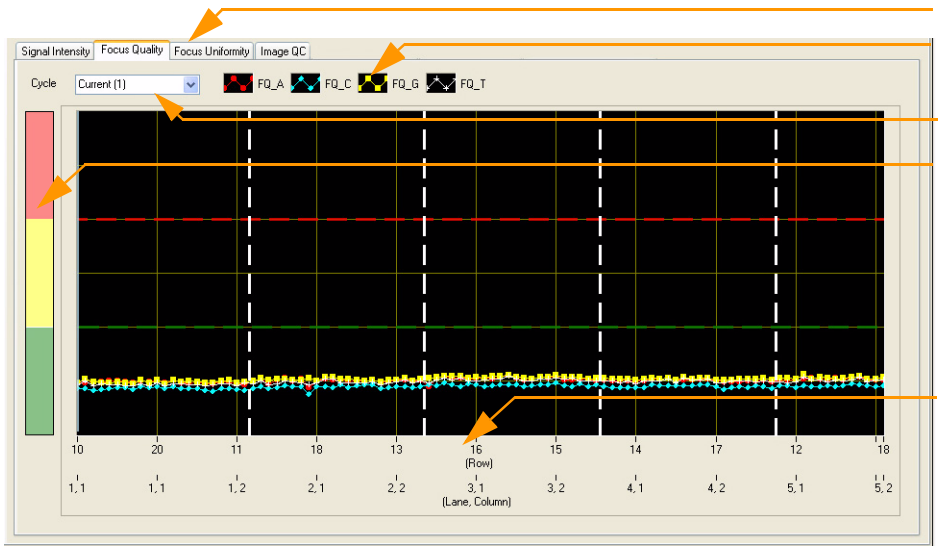


Figure 102 Analysis Viewer Screen

Scaled Versus Raw Values

The overview display shows the quality metrics data in two ways:

- **Scaled display**, in which a color bar is displayed on the left to indicate "good" values in the green region, "marginal" values in the yellow region and "failed" values in the red region. The thresholds of these regions are set in IPAR for some quality metrics (see *Thresholds Scaled Overview Display* on page 188). Yellow and red values can be higher or lower than green values, depending on the settings for the particular metric.
- **Raw value display**, in which the raw values of quality metrics are plotted, without color bar.

Zooming In and Zooming Out

Analysis Viewer has the following zoom options:

- **Zoom in** by holding the Shift key down, left-clicking and dragging the mouse to select an area and then releasing the mouse key. See Figure 103 for a zoomed in view of an area from roughly the middle of the red region through the middle of the green region and from lane 2, column 1, row 20 through lane 4, column 2, lane 20.

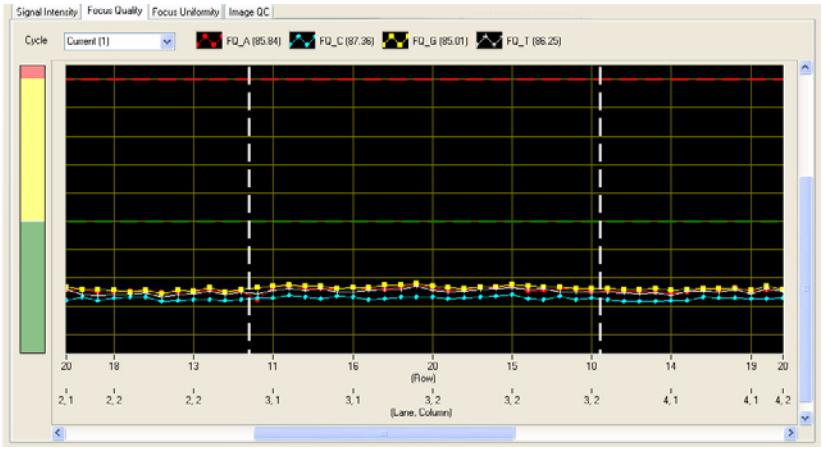


Figure 103 Zoomed in view Analysis Viewer

Scroll bars appear that allow maneuvering through the complete graph. A user can zoom in again by using the same procedure.

- To zoom out, right-click on the graph to bring up the context menu (Figure 104) and select **Zoom out**. If you have zoomed in two or more times, **Zoom Out All** will be enabled, which will return the graph to the original un-zoomed state.

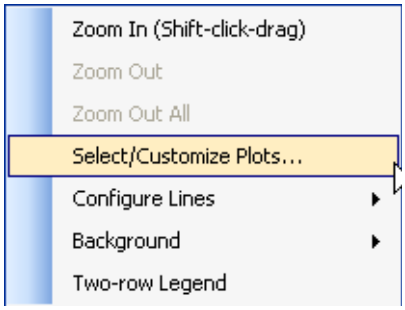


Figure 104 Analysis Viewer context menu

NOTE

The **Zoom In** menu item in the context menu provides instruction on how to select an area and zoom using the short cut keys. Unlike all other menu items in this context menu, this menu item is there solely to provide instruction.

Customizing Plots

Click on **Select/Customize Plots** in the context menu (Figure 104) to bring up the **Show/Hide/Customize Plots** dialog box (Figure 105).

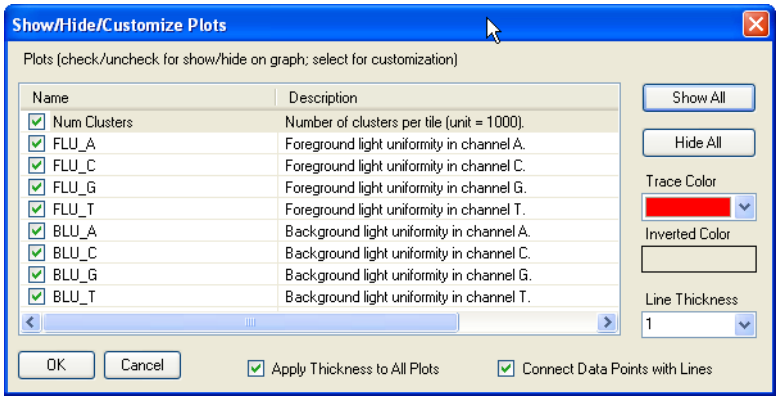


Figure 105 Select plots in Analysis Viewer

- To hide a plot on the graph, deselect the check box. The graph will automatically rescale if necessary. To show a plot, select the check box. Again, automatic rescaling will occur.
- To show all the plots on the graph click the **Show All** button.
- To hide all the plots click the **Hide All** button.
- To change the color of a plot line and points, select the plot and select a color from the **Trace Color** dropdown menu.
- To change the thickness of all the lines, select the **Apply Thickness to All Plots** checkbox and select a thickness from the **Line Thickness** dropdown menu.
- To change the thickness of an individual plot line, deselect the **Apply Thickness to All Plots** checkbox, select the plot, and select a thickness from the **Line Thickness** dropdown menu.

- To just show the points in the plots rather than have connecting lines, deselect the **Connect Data Points with Lines** check box.

NOTE

Single-clicking a legend item in the overview display will highlight the line (thickens the plot line).

Cycle Selection

By default IPAR displays QC data from the latest imaging cycle. To view tile data for previous cycles, select a cycle from the **Cycle** dropdown box (Figure 106). This can be done in real time as data is sent to the control. You can return to the current cycle by selecting **Current (<n>)**.

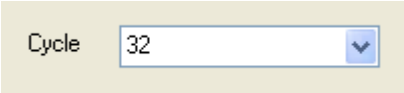


Figure 106 Cycle Selection in the Analysis Viewer

Viewing Raw Values in the Overview Display

You can view raw values in the overview display using the vertical Y-value line.

- Click anywhere in the graph to bring a light blue vertical line to that click-location.
- If the mouse button is held down at particular tile of interest, the values for all parameters shown on the overview tab of interest will be displayed in a transparent label next to the vertical Y-value line as show below (Figure 107). Logical coordinates of the tile of interest on the flow cell are also displayed.

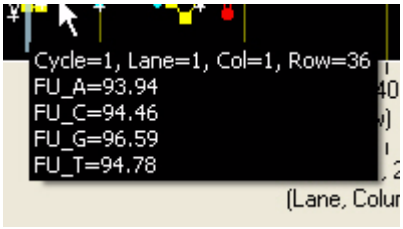


Figure 107 Plot value line

- The vertical Y-value line can be dragged to display values for different tiles.
- The vertical Y-value line can be hidden, shown, or customized using the context menu (Figure 108).

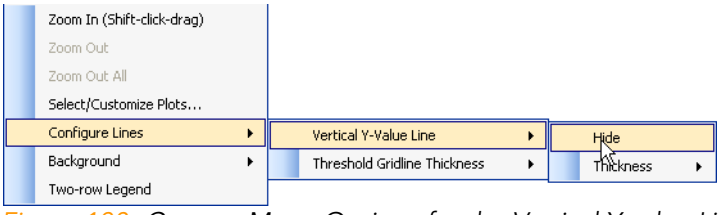


Figure 108 Context Menu Options for the Vertical Y-value Line

Additional Context Menu Options

The context menu has a few more options to customize your overview display:

- You can set the background to white as shown in Figure 109.

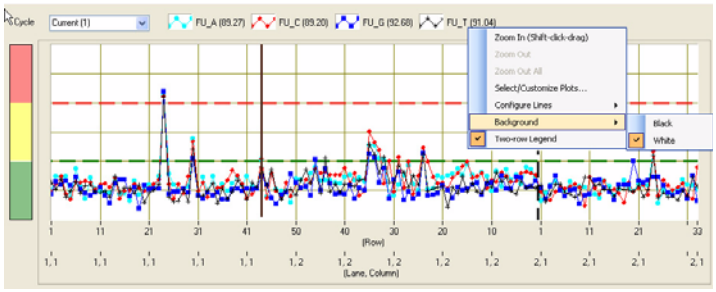


Figure 109 Setting White Background

- You can set the thickness of the threshold gridlines as shown in Figure 110.

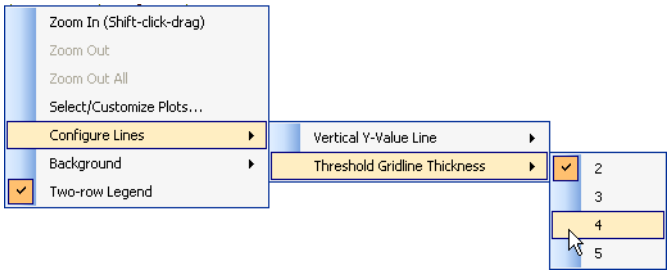


Figure 110 Setting the Thickness of the Threshold Gridlines

- You can set a two-row legend as shown in Figure 111.

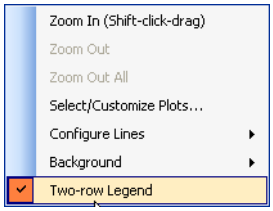


Figure 111 Setting the Two-row Legend

Individual Parameter Plots

The legend of the overview display is at the top above the graph (Figure 112), and enables selecting individual parameter plots. To do this for a specific quality metric, double-click the legend item.

Figure 112 Analysis Viewer Legend

This will bring up a "raw" view of the un-scaled values (Figure 113). If done in real time as data are being added to the control, this view will update as data from the next analyzed tile becomes available.

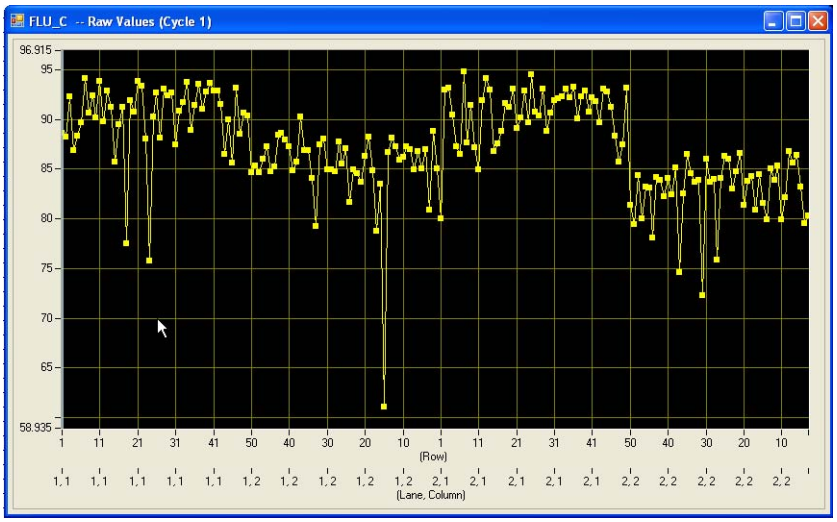


Figure 113 Individual Parameter Plot

Quality Metrics in IPAR

Using IPAR, you can monitor several quality metrics during a run on the Genome Analyzer. These quality metrics are organized in tabs in the overview display of the Analysis Viewer. The meaning of the quality metrics is explained below.

Quality Metrics Explanation

The following quality metrics help you evaluate cluster quality during a run. Many perform a statistical operation on an individual image channel (A, T, C, G) during a given cycle.

Table 24 Quality Metrics in Analysis Viewer

Tab	Legend Item	Description
Signal Intensity	Q87_N	The value of the 87.5 percentile signal intensity (all clusters) of the selected channel, per tile.
Focus Quality	FQ_N	Image focus quality of the selected channel, per tile. It represents the sharpness or focus quality of the image. A high-quality image will have a focus metric of 80 - 85; focus quality below 75 becomes a concern.
	FU_N	Image focus uniformity of the selected channel, per tile. 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 homogeneous focus have focus uniformity numbers of about 90–95.
Image QC	Num Clusters	Number of clusters per tile (unit = 1000). This parameter is not channel specific.
	FLU_N	Foreground light uniformity of the selected channel, per tile.
	BLU_N	Background light uniformity of the selected channel, per tile.

Thresholds Scaled Overview Display

The thresholds of the Y-axis color bar in the scaled overview display are stored in the xml file QCmetrics.xml, located in the folder C:\Illumina\SCS2.x\DataCollection\bin\config.

The default values are shown below:

Table 25 Thresholds for the Y-axis Color Bar in the Overview Display

Legend Item	Description	Green	Yellow	Red
FQ_N	Image focus quality	100-70	70-60	60-50
FU_N	Image focus uniformity	100-90	90-70	70-50

Storage of IPAR Data

IPAR analysis generates output results which are saved on the instrument computer or the IPAR server. On both computers the results are saved in the RunFolder\Data directory created for the run in progress.

IPAR produces the following analysis results:

Computer	Subfolder	Key Files/Folders	Description
Instrument computer	D:\Runs\RunFolder\Data	Run Browser folder	Contains *.bro files used with the Run Browser application. Each file contains run quality metrics in one file per lane, per cycle.
IPAR server	E:\Analyzed\RunFolder\Data	IPAR folder	Contains IPAR image analysis results. This folder is saved on the IPAR server under <Run Folder>\Data. This Run Folder is created on the IPAR server when a run is started, and is given the same name as the Run Folder on the instrument computer.
IPAR server	E:\Analyzed\RunFolder\Data	.params file	Contains run and analysis-specific information.

Network Copy Options

Images and IPAR analysis data from each experiment can be moved to a dedicated network location. The software provides user-configurable flexible data management options. In all of the options described below IPAR analysis data will be saved locally and transferred to a specified network location.

There are three supported scenarios for IPAR data and results management:

- IPAR saves images on the IPAR server and transfers the images to specified network location
- Images are saved only to the IPAR server (they are not moved to a network location)
- Instrument computer saves images

Once the data management script has completed all data copy and image copy at the end of the run, it generates a special tag file in the network run folder, GA_Netcopy_Complete.txt. This file can be used as a flag to start the offline analysis.

The sections below explain the three options, including the required settings for flags in configuration files.

IPAR Saving and Transferring Images

IPAR saves images on the IPAR server and transfers the images to specified network location.

IPAR analysis data and images from the experiment get transferred to a specified network location (default option).

IPAR saves images locally and on the specified network location as they are generated. In case images were not saved on the network during the current cycle, the instrument computer will copy them during the next imaging cycle. Images and results need to be saved to the same network destination.

In this scenario, the IPAR server has the primary responsibility for saving images to the network run folder and the instrument run computer functions as a fail-safe in case the IPAR server does not copy all images.

This is the preferred option for SCS2.01/IPAR1.01.

Configuration for IPAR Saving and Transferring Images

To configure the system for this scenario, open the configuration files in Notepad, set the indicated elements, and save under the same name in the same location. The required values are marked in **bold**.

- For GalaxyRunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:

```
<EnableNetworkCopy>true</EnableNetworkCopy>
<CopyImageFiles>true</CopyImageFiles>
```

```
<NetworkCopyRootFolder>
          ENTER_PATH_TO_SERVER_ROOT_FOLDER</
          NetworkCopyRootFolder>
<DeleteFilesAfterNetCopy>true</
          DeleteFilesAfterNetCopy>
<CopyGoldcrestRunBrowserData>true</
          CopyGoldcrestRunBrowserData>
```

- For ImagePath.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `SaveClusterImages` element:

```
- <ImagePath SaveFocusImages="false"
          SaveFinalFocusImage="false"
          SaveClusterImages="true">
```

- For RunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `OnlineAnalysis On` element:

```
<OnlineAnalysis On="true" StopRunOnError="false" />
```

- For Analysis.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:

```
<Results Compress="true" AutoArchive="true"
          ArchiveRoot="ENTER_PATH_TO_SERVER_ROOT_FO
          LDER" />
```

- For StarGazerShell.xml in the folder C:\Illumina\IPARx.x on the IPAR computer, set the `Images` element:

```
<Save ClusterDetails="true"
          ImageAnalysisResults="true"
          Images="true" />
```

Images Not Transferred

Images are saved only to the IPAR server (they are not moved to a network location)

Only IPAR analysis data gets transferred to a specified network location, images will not be transferred in this scenario.

Configuration for Images Not Transferrred

To configure the system for this scenario, open the configuration files in Notepad, set the indicated elements, and save under the same name in the same location. The required values are marked in **bold**.

- For GalaxyRunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:

```
<EnableNetworkCopy>true</EnableNetworkCopy>
<CopyImageFiles>false</CopyImageFiles>
<NetworkCopyRootFolder>
          ENTER_PATH_TO_SERVER_ROOT_FOLDER</
          NetworkCopyRootFolder>
<DeleteFilesAfterNetCopy>false</
          DeleteFilesAfterNetCopy>
<CopyGoldcrestRunBrowserData>true</
          CopyGoldcrestRunBrowserData>
```

- For ImagePath.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `SaveClusterImages` element:

```
- <ImagePath SaveFocusImages="false"
          SaveFinalFocusImage="false"
          SaveClusterImages="false">
```

- For RunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `OnlineAnalysis On` element:
```
<OnlineAnalysis On="true" StopRunOnError="false" />
```
- For Analysis.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:
```
<Results Compress="true" AutoArchive="false"
          ArchiveRoot="ENTER_PATH_TO_SERVER_ROOT_FO
          LDER" />
```
- For StarGazerShell.xml in the folder C:\Illumina\IPARx.x on the IPAR computer, set the `Images` element:
```
<Save ClusterDetails="true"
          ImageAnalysisResults="true"
          Images="false" />
```

Instrument Computer Saving Images

The instrument computer saves images

IPAR does not save images, only analysis data.

Configuration for Instrument Computer Saving Images

To configure the system for this scenario, open the configuration files in Notepad, set the indicated elements, and save under the same name in the same location. The required values are marked in **bold**.

- For GalaxyRunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:
```
<EnableNetworkCopy>true</EnableNetworkCopy>
<CopyImageFiles>true</CopyImageFiles>
<NetworkCopyRootFolder>
          ENTER_PATH_TO_SERVER_ROOT_FOLDER</
          NetworkCopyRootFolder>
<DeleteFilesAfterNetCopy>true</
          DeleteFilesAfterNetCopy>
<CopyGoldcrestRunBrowserData>true</
          CopyGoldcrestRunBrowserData>
```
- For ImagePath.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `SaveClusterImages` element:
```
- <ImagePath SaveFocusImages="false"
          SaveFinalFocusImage="false"
          SaveClusterImages="true">
```
- For RunConfig.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the `OnlineAnalysis On` element:
```
<OnlineAnalysis On="true" StopRunOnError="false" />
```
- For Analysis.xml in the folder C:\Illumina\SCSx.x on the instrument computer, set the following elements:
```
<Results Compress="true" AutoArchive="false"
          ArchiveRoot="ENTER_PATH_TO_SERVER_ROOT_FO
          LDER" />
```
- For StarGazerShell.xml in the folder C:\Illumina\IPARx.x on the IPAR computer, set the `Images` element:
```
<Save ClusterDetails="true"
          ImageAnalysisResults="true"
          Images="false" />
```

Network Copy Configuration Summary

The configurations to set up the supported scenarios for network copy are summarized for the expert user in Table 26 on page 193 and Table 27 on page 193.

Table 26 Elements to Be Changed in the Configuration Files

Configuration File	Flag	*IPAR Saving and Transferring Images*	*Images Not Transferred*	*Instrument Computer Saving Images*
GalaxyRunConfig.xml	EnableNetworkCopy	True	True	True
GalaxyRunConfig.xml	CopyImageFiles	True	False	True
GalaxyRunConfig.xml	NetworkCopyRootFolder	Valid path	Valid path	Valid path
GalaxyRunConfig.xml	DeleteFilesAfterNetCopy	True	False	True
GalaxyRunConfig.xml	CopyGoldcrestRunbroData	True	True	True
ImagePath.xml	ImagePath SaveClusterImages	True	False	True
RunConfig.xml	OnlineAnalysis On	True	True	True
Analysis.xml	Results AutoArchive	True	False	False
Analysis.xml	Results ArchiveRoot	Same valid path as NetworkCopyRoot Folder	Same valid path as NetworkCopyRoot Folder	Same valid path as NetworkCopyRoot Folder
StarGazerShell.xml	Save Images	True	False	False

Table 27 Configuration File Locations

Configuration File	Computer	Location
GalaxyRunConfig.xml	Instrument computer	C:\Illumina\SCSx.x
ImagePath.xml	Instrument computer	C:\Illumina\SCSx.x
RunConfig.xml	Instrument computer	C:\Illumina\SCSx.x
StarGazerShell.xml	IPAR server	C:\Illumina\IPARx.x
Analysis.xml	Instrument computer	C:\Illumina\SCSx.x

Pipeline Analysis of IPAR Data

Image analysis data generated with IPAR can be processed further with the Genome Analyzer Pipeline Software (Pipeline) version 1.0 or later. The Pipeline will perform base calling and alignment after calculating the cross-talk matrix, phasing and pre-phasing values for the experiment.

To perform Pipeline analysis of IPAR data, ensure the following steps have been taken:

- You have installed Pipeline v1.0 on the off-line server—earlier versions of the pipeline are not compatible with IPAR output.
- The experiment run folder containing the IPAR image analysis results folder has been copied to the off-line server.
- The params file for the experiment has been copied to \RunFolder\Data on the off-line server.

If you are using all mechanisms for data transfer provided by Illumina, the second and third conditions will be always met.

See the *Genome Analyzer Pipeline Software User Guide* for instructions on how to further analyze IPAR image analysis data with Pipeline.

Appendix A
Run Folders

Topics

Introduction

Each run generates a run folder that contains data files and log files specific for that run. All run folders are stored in a single folder (see *Run Folder Path* on page 196).

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 add the flow cell ID to the run folder name. 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 28 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 the 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.
	*.params file <run_name>.params	Identifies the name of the instrument.

Table 28 Run Folder Contents (Continued)

Subfolder	Key Files/Folders	Description
	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.
	PairedEndInfo.xml	At the beginning of a paired-end run, this file is created. It contains the FirstRead\Length attribute indicating the "turnaround point" of the paired-end run and the length of the first read.
Images	Contains one folder for each lane. Each lane folder contain one subfolder for each cycle.	Each cycle folder contains TIFF files for each lane, named in the format of <sample>_<lane>_<pos>_<base>.tif
	Focus folders (optional)	Contains the TIFF focus images.
Data	Run Browser folder	Contains *.bro files used with the Run Browser application. Each file contains run quality metrics in one file per lane, per cycle.
	IPAR folder	Contains IPAR image analysis results. This folder is saved on the IPAR server under <Run Folder>\Data. This Run Folder is created on the IPAR server when a run is started, and is given the same name as the Run Folder on the instrument computer.
	.params file	Contains run and analysis-specific information.
AnalysisLogs	Contains QC files (one per cycle). Values in these files are displayed in the Analysis Viewer for each cycle.	Each XML file is named as follows: <QC_tab_name>CycleNumber.xml. For example, FocusQuality15.xml contains focus quality information for all tiles of cycle 15.
Config		Contains configuration parameters for the run that are stored in XML files.
IPAR_Config		Contains configuration parameters for the current run on the IPAR server that are stored in XML files.
ReadPrep1	Contains Images and Data subfolders specific to the preparation of Read 1	Contains quality results from Read 1 <ReadPrep> cycle processed by IPAR.
ReadPrep2	Contains Images and Data subfolders specific to the preparation of Read 2	Contains quality results from Read 2 <ReadPrep> cycle processed by IPAR.
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 fields 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 Analyzer 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**.

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.

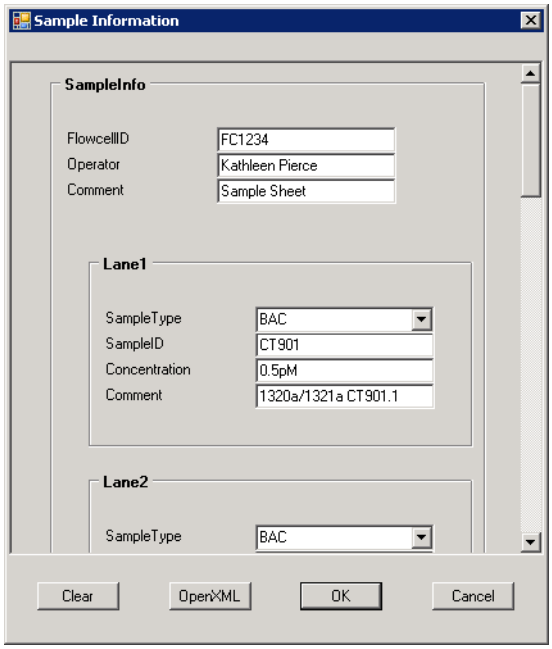


Figure 114 Sample Sheet Editor

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. If Required="true" in the RCMConfig.xml, the following things happen:
  - **a.** The software searches for the sample sheet in the sample sheet folder on the network with the provided flow cell ID and copies it to the run folder.
  - **b.** If the sample sheet is not found then a browse dialog pops up for the user to browse the appropriate sample sheet.

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, and Blocking-CyclicPumping. Some Genome Analyzer chemistry definitions are Prime, FirstBase, and CompleteCycle.
- **Protocol**—Invokes chemistry definitions in a particular sequence to perform the run. Genome Analyzer sequencing protocols perform image data acquisition steps (incorporation) 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 cycles. You can shorten the run by limiting the number of tiles imaged, although this generates concomitantly less data.
- **Incorporation**—This portion of the sequencing protocol calls out for the imaging of the current cycle, then calls out the chemistry for the removal and washing of the fluorescent bases and the subsequent addition of the next base in the sequence.

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 is placed 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 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.

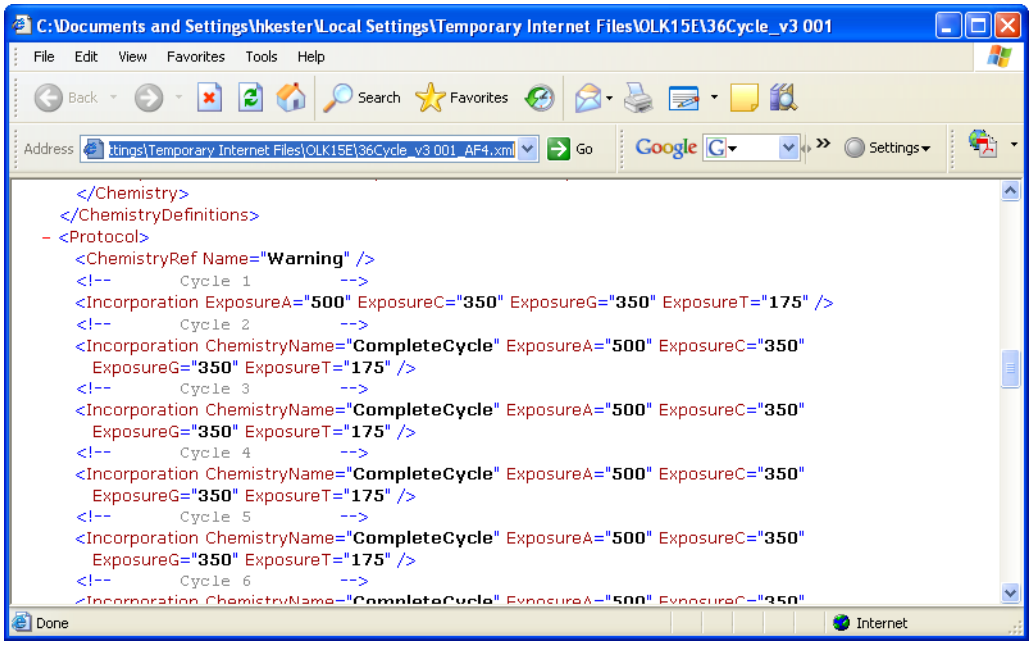


Figure 115 Protocol Section of Sequencing Recipe File

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 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.

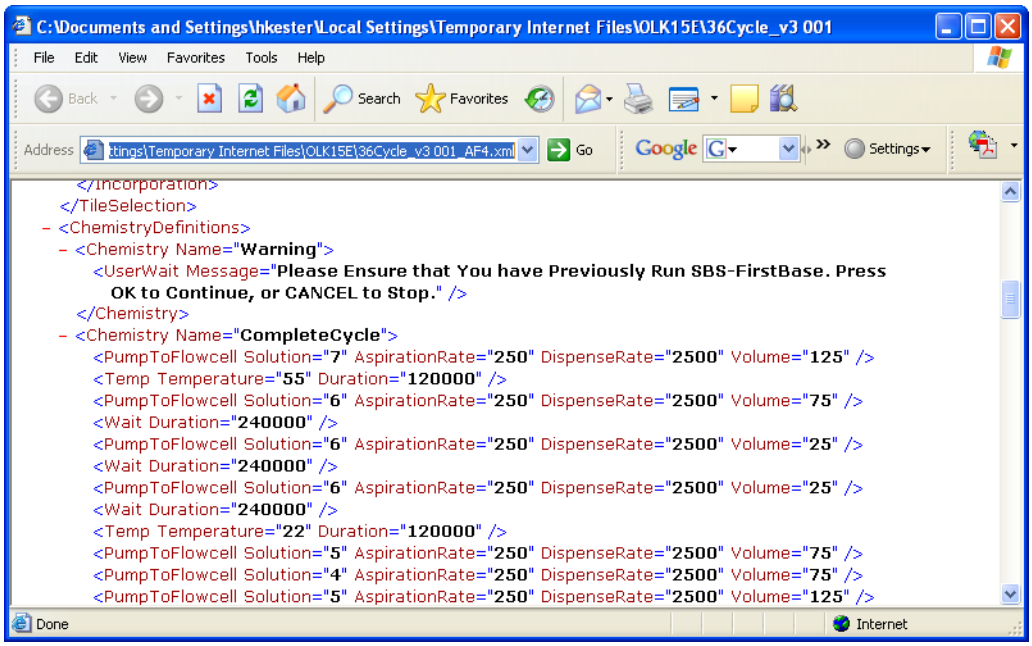


Figure 116 Chemistry Definition Section of Sequencing Recipe File

General Commands

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

Table 29 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 29 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. Click **OK** to continue the run or **Cancel** to stop the run.

Cluster Station Commands

These commands appear only in Cluster Station recipes.

Table 30 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 31 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.
RowRange	**Min**—The first row in the range {1–50} **Max**—The last row in the range {1–50}	Identifies a range of adjacent rows for incorporation.
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. LaneRange only applies to the first four lanes.
Lane	**Index**—The number of the lane for incorporation	Identifies one or more lanes for incorporation. 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	Identifies one or more rows for incorporation. Write separate <Row Index= ...> commands for each lane.
ReadPrep	**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.	Applies chemistry and performs test scan with separate online analysis before each paired-end read. The second occurrence of this element marks the "turn around" point of the paired-end run and determines the length of the first read.

ReadPrep Cycles

In addition to Incorporation and Cleavage, the ReadPrep element is a third cycle type in the protocol section of the SCS recipes. The ReadPrep cycle performs scans and collects data before each paired-end read. The second occurrence of this element marks the "turnaround point" of the paired-end run and determines the length of the first read. ReadPrep does not affect cycle numbers assigned by the software to any Incorporation or Cleavage elements.

Each ReadPrep cycle is processed by the IPAR server as the first cycle of a new run. Corresponding results on the IPAR server are written to separate sub-folders of the Run Folder, "Read 1" and "Read 2." After the second ReadPrep element, the main run resumes as if no ReadPrep cycles existed in the recipe.

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 for Incorporation. All lanes within those rows are selected by default, unless the LaneRange command is used to reduce the number.

```
<TileSelection>
    <Incorporation>
      <RowRange Min="6"  Max="10" />
      <RowRange Min="21" Max="25" />
      <RowRange Min="36" Max="40" />
    </Incorporation>
</TileSelection>
```

Reducing the Number of Lanes

This selection chooses only the middle four lanes for incorporation imaging.
<TileSelection>

```
    <Incorporation>
      <Lane Index="3"/>
      <Lane Index="4"/>
      <Lane Index="5"/>
      <Lane Index="6"/>
    </Incorporation>
</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 lane.

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

Tiles 23–27, 33–37, 43–47 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" />
```

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

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

In Lane 4, however, only 5 rows will be imaged.

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

End of Incorporation section.

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

```
<Incorporation>
  <Row Range Min="1" Max "35">
</Incorporation>
```

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 and 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 Mix.

```
<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.

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, and incorporation. The exact combination is determined by the instrument and the recipe.

This sample protocol from the Genome Analyzer consists of 36 cycles of incorporation followed by imaging. The Incorporation command takes two inputs, an optional chemistry and exposure times for each filter/base.

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

Displays a warning that you first have to run the SBS-FirstBase protocol.

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

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

```
<Incorporation ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />
```

Image incorporation tiles, exposure times: 500 ms, 350 ms, 350 ms, and 175 ms per picture for channels A, C, G, and T, respectively.

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

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

```
<Incorporation ChemistryName="CompleteCycle"
       ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />
```

Call up CompleteCycle protocol, image incorporation tiles.

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

Repeat.

```
<Incorporation ChemistryName="CompleteCycle"
       ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />

<!--      Cycle 4       -->

<Incorporation ChemistryName="CompleteCycle"
       ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />

.

.

.

<!--     Cycle 35 -->

<Incorporation ChemistryName="CompleteCycle"
       ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />

<!--     Cycle 36-->

<Incorporation ChemistryName="CompleteCycle"
       ExposureA="500" ExposureC="350"
       ExposureG="350" ExposureT="175" />

<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 three hours to linearize, block, and hybridize the primers before sequencing.

A paired-end flow cell can not be stored after the blocking step.

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 Station 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 224.

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 paired-end sample prep kit?

Illumina recommends 1–5 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 template size is determined by the length of the insert of interest. The size range is 200 ±8 bp.

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 6 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 Genome Analyzer Pipeline software 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 200 ±8 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 PhiX, we recommend 1 pM final concentration. This yields about 85,000 clusters per tile on a 1.4 mm flow cell.

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 lanes.

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 1.5 hours, assuming that you image all 100 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.

How can I prevent cross-contamination between buffers?

Be careful when preparing reagents for the Genome Analyzer. Use filter tips to prevent contamination between buffers.

Controls

Illumina recommends running a control 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 sample 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 Genome Analyzer 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 PhiX sample?

Currently, the PhiX sample for paired reads is only available in the paired-end installation and training kit.

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. 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 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 207.

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 FirstBase_v<#>.xml. For the remaining rounds use 36Cycle_PE_GA2_v.1.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.

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 124.

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 124.

Flow Cells

What is a tile?

A tile is an imaginary square within a lane, measuring 760 µm x 720 µm on each side (1.4 mm flow cell). Each of the eight lanes in a flow cell is subdivided into two columns of 50 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.

I have used an earlier Genome Analyzer system before, but the tile size for the Genome Analyzer II has changed. What does this mean for the cluster density?

The surface area for each tile of the Genome Analyzer II is 4.7 times bigger than that for the earlier system. As a consequence, the lower and upper limits for cluster density (total clusters) have changed:

	Genome Analyzer II with 1.4 mm flow cell	Preceding Genome Analyzer system with 1.0 mm flow cell
Tile size	0.57 mm^2/tile	0.12 mm^2/tile
Lower limit cluster density	104,000 clusters/tile	22,000 clusters/tile
Upper limit cluster density	132,000 clusters/tile	28,000 clusters/tile

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 or 2. Any other options will generate an error.

To change the number of tiles imaged, see *Configuring Tile Selection* on page 213.

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

See *Completing Read 1* on page 139 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 117.

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

Cluster Station, Paired-End Module, and Genome Analyzer recipes are optimized in a range from 15 µl/min to 250 µl/ min depending on the hardware and the chemistry step. The maximum limits are 250 µl per minute. Do not use more on the Genome Analyzer or it will cavitate and introduce a lot of bubbles into the system.

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. Volumes above 250 µl automatically pull more than 2 syringe strokes.

Why are there eight syringes if they are controlled together?

Using a separate syringe for each lane 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?

Isolated small bubbles are not a problem but large bubbles can affect the flow rate across the flow cell. Strings of small bubbles can also be problematic.

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.

Quality Metrics

Most of the values in the QMD report are zero, does this mean my sequencing run was unsuccessful?

Zeros are expected for the most of the fields in the report.

IPAR

What are the supported network configurations for IPAR?

The supported network configurations are described in the *Sequencing Site Preparation Guide for Cluster Station, Genome Analyzer, IPAR, and Paired-End Module*.

How do I make sure IPAR is enabled?

In the DataCollection\Bin\Config folder, open the file RunConfig.xml and verify that the OnlineAnalysis element is set on="true". If this is not the case edit the element to be set on "true".

How do I start IPAR analysis?

IPAR will start automatically as long as you have an active log in session on the IPAR computer. There is a "Startup" process running on IPAR that listens for a connect request from the GA computer. Each time a recipe is started or resumed the GA will automatically initiate a new IPAR session.

What is the startup initialization sequence for turning on the instrument, the GA computer and the IPAR computer?

In general, there is no requirement to turn on the instrument, GA computer and IPAR computer in any given order. They all need to finish booting up before starting a run, and there must be active log in sessions on the GA and IPAR computers.

Do I need to reboot the IPAR computer between runs?

No, this is not required. If you experience any connection problems when starting a run, it is best to log out of the GA and IPAR computers, and log back in. This will free all the resources and allow the network port connections to be re-established normally. If this doesn't solve the connection issues, then you can reboot both computers and try again. There are icons on the desktop of the IPAR computer for shutting down and for restarting the IPAR computer.

Do I need to reboot the GA computer between runs?

Not under normal circumstances. However you should close and restart SCS 2 on the instrument computer and restart it before starting a run, just to make sure there will be sufficient resources to complete the run.

Do I need to clean up data from previous runs manually on the IPAR computer?

Yes, just as you need to do so periodically on the GA instrument computer. Run data accumulates on the IPAR storage array and needs to be moved or deleted periodically. The array holds approximately 3.2 Terabytes of data, which can hold roughly 10 runs of data analysis results if images are not being saved, and 3 runs if images are being saved. We recommend cleaning out the E:\Analyzed folder (which is the main folder on the storage array) every few runs to make sure there is sufficient space for the next runs.

How can I tell if IPAR is working?

There are three ways to tell. The easiest is to watch the tile layout graph during the imaging cycle. When all four images have been acquired for a tile, that tile turns blue on the graph. When IPAR has processed the tile, it will turn green, yellow or red according to its QC score. If the tiles stay blue, then IPAR is not actively analyzing the run. The second indication is that the analysis window has a graph of the various QC metrics. If data points are being added to the graph, then IPAR is active. Finally you can open a remote desktop window to the IPAR computer and see that it has a command window open where the processed tiles are listed.

What should I do if IPAR doesn't start up automatically?

If the network is not configured properly, the cable is disconnected, the IPAR server is off, or Startup.exe is not running on the IPAR computer, you will get error messages when SCS 2 starts up. Correct the identified problem.

If SCS 2 does not display an error, then starting IPAR is disabled in RunConfig.xml. Verify that IPAR is configured to start: in the RunConfig.xml file on the instrument PC, verify that Analysis enabled="true".

Is it necessary to connect to the IPAR server with Remote Desktop?

It is required to be logged in to the IPAR server for the IPAR analysis to be able to start up. You can either use the KVM switch provided in the IPAR chassis to share Keyboard/Video/Mouse with the GA computer, or you can attach your own Keyboard/Video/Mouse to the IPAR chassis. Lastly, you can use Remote Desktop to login remotely from the instrument PC to IPAR. Remote Desktop is the easiest way, since it doesn't require the IPAR chassis to be next to the instrument. It is also possible to put a monitor, keyboard and mouse directly on the IPAR server, wherever it is located.

If IPAR analysis encounters an internal error, will it cause me to lose the run?

Not if the following settings are used: StopRunOnError="false" in RunConfig.xml, found in the DataCollection\Bin\Config folder. Edit RunConfig.xml and find the OnlineAnalysis element. Set StopRunOnError="false" so that IPAR errors won't cause the run to stop. Make sure network copy is enabled on the GA computer. That way the images will be copied to the offline analysis server and you won't lose any run data.

Can you explain the red/green/yellow of the tiles?

When IPAR analyzes the images for a tile for the current cycle it sends back the values for each quality parameter to the instrument computer. The GA software compares them to the established QC thresholds. If all of the values are within normal expected ranges, the tile will be colored green, meaning "good." If any of the values are in the marginal range, then the tile is colored yellow, meaning "marginal." If any of the values are outside the valid range, then the tile is colored red, meaning "bad." Not all parameters are scaled to have good/marginal/bad ranges. Some parameters are simply data that gets graphed without a QC score. Note that the QC thresholds are contained in a configuration file, but the values have been pre-configured by Illumina to cover standard protocols.

What happens if I stop the recipe and then resume it?

The IPAR session is refreshed, and the run will continue with IPAR analysis enabled automatically.

What happens if the SCS 2 has an error and stops? Will I be able to restart the software and get the run going again with IPAR enabled?

In this case, close SCS 2 and log out of the GA computer or reboot the GA computer. When you restart SCS 2, you will need to reestablish the focal plane, as usual. Then you will open the recipe from the

run folder and find the recipe step where the run left off. Click that step in the protocol panel and then click Resume. The IPAR session will refresh and the run will continue with IPAR enabled. However you cannot run any other recipes (e.g. a wash or test recipe) before you complete the current one because you will invalidate the IPAR session and the resume will fail.

What happens if the IPAR software has an error or exception and stops analyzing? Can I restart IPAR and have it catch up?

The default setting is to allow data collection to continue normally even if the IPAR software has an error and stops. You won't get any new tile information displayed in the analysis graphs, but the run will not be affected. The IPAR software cannot be restarted to have it catch up to the data collection. However, if you stop the run in the imaging cycle where IPAR had the error, you can resume that imaging cycle and the IPAR session will be refreshed and will begin processing the images. But you cannot stop it at a later cycle and resume, because IPAR will fail due to the missing cycle data.

Do I have to worry about computing the default offsets on the IPAR?

IPAR is calculating the offsets throughout the experiment in progress. It compares the offsets values for initial cycles with the values generated from the previous experiment. If the difference is above predefined threshold, IPAR will reanalyze all data collected so far with the new offsets and will use current offsets for the rest of the cycles.

The default offsets are located in the bin\config\Analysis.xml file on the client PC. IPAR will update them automatically when they change. There is also an offsets log created in the run folder, which lists the observed offsets for each cycle.

Do I still get a report for the initial first base recipe?

Yes, when the first base recipe is complete the Runbrowser will automatically generate and display the first base summary report.

Can I still use Runbrowser with IPAR?

Yes, the IPAR analysis software will create the *.bro files. They are compatible with the Runbrowser and you can view all the same information as before.

Can I run the Pipeline on the IPAR system?

No, the Pipeline only runs on specially configured Linux systems.

Can I get the IPAR software and run it on my own computer?

The IPAR software has been highly tuned to run on specific hardware that is optimized to receive images from the instrument computer and perform analysis in real-time. Illumina does not validate or support the use of the IPAR software on any system other than the IPAR hardware.

Will the image analysis results from IPAR be different from the results of the offline analysis Pipeline?

No, at least not significantly. IPAR and Pipeline v1.0 use the same image analysis algorithms with math libraries for the corresponding operating system they are designed for (IPAR - for Windows, Pipeline v1.0 - for Linux). Since the math libraries are slightly different on the two platforms, there are minute, statistically insignificant differences in result calculations. Both platforms use the same algorithms, but they are compiled with different compilers and libraries.

What data is created during an IPAR analysis?

IPAR analysis creates data both on the GA computer and the IPAR computer.

On the GA computer, the tile parameters that were plotted during the run are accessible in the run folder, in the following location: RunFolder\AnalysisLogs subfolder. They are saved in XML format. In addition the Runbrowser files and the standard GA run logs are created for use with the Runbrowser application. The images are saved on both the GA computer and locally until they are moved off to the network server.

On the IPAR computer, the IPAR run folders are located under the E:\Analyzed\RunFolder\Data root folder. The image processing results are created in the IPAR run folder that has the same name as the one being created for this run on the GA computer. Note that the image analysis files created by IPAR are compatible with the Pipeline v1.0 input requirements for base calling.

Can I still save my raw images if I am using IPAR?

Yes. The supported mechanism is to use the SCS event scripts to move the images to the network server for offline analysis with the Pipeline. The GA computer will have enough space on the D: drive (the RAID array) to hold all images and logs for one full 36 cycle run. Longer runs will require using the network copy feature to delete the images after they have been successfully copied to the network.

I got an error message when I started a run indicating something like "only one usage of the port is allowed" What do I do?

Log out of both computers and log back in. This will ensure that Windows relinquishes the port resources so they are available for the new run.

Does the IPAR chassis have to be adjacent to the GA instrument?

Co-location of IPAR and GA is strongly recommended but is not an absolute requirement. If they are co-located then it is possible to use the KVM switch in the chassis to share the keyboard, video monitor and mouse between the GA and the IPAR computers. In addition the GA, the instrument computer and the paired end module can be plugged into the IPAR UPS (there are three available plugs). Finally, it avoids long runs of Ethernet cables.

How does IPAR connect to the GA computer?

The GA computer requires an additional Network Interface Card (NIC) to connect to the IPAR computer. This is provided by Illumina. We typically provide a dual channel NIC, for future expandability, but only use the first port (the one on the left when looking at the computer from the rear). The IPAR computer comes with two network connections, accessible from the back of the chassis. The upper one connects to the GA computer, and the lower one connects to the Local Area Network (LAN).

Do I need a crossover Ethernet cable to connect the GA to the IPAR?

We require a 1 gigabit per second (1gbps) link between the instrument computer and the IPAR server. The standard for such connections indicates that the link level hardware be auto-sensing, so that either a normal cable or a crossover cable can be used. The cables must meet the full Cat 5e specification, with 4 data pairs. These cables are provided by Illumina, but if longer ones are required, they can be bought off the shelf as long as they meet the full Cat 5e specification.

During analysis I got the following notification: "Image analysis offsets for this experiment were recalculated." – What does it mean?

The difference between the channel offsets in the IPAR configuration file and the offsets for the experiment in progress exceeds predefined thresholds. Therefore data acquired so far will be re-analyzed using the offsets values from the current run. All subsequent cycles will be analyzed with the offsets for the current run as well.

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 1500 bp. 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 over-sampling should be adequate. For targeted resequencing involving mixes of

many PCR products, 75-fold over-sampling 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 1–5 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 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 lane. We do not know how much of this binds to the surface, but this amount is enough to visualize around 13 million clusters in one lane.

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 com-

mon 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.

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?

To process the data from a single run, the system requires 500 GB to 1 TB (depending on factors such as cycles, cluster density). Images are acquired and stored on the instrument workstation and must then be transferred to an external computer to be analyzed by the Genome Analyzer 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 Genome Analyzer 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.

Index

F

G

H

I

Q

R

S

T

U

V

W

X

Y

Z

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