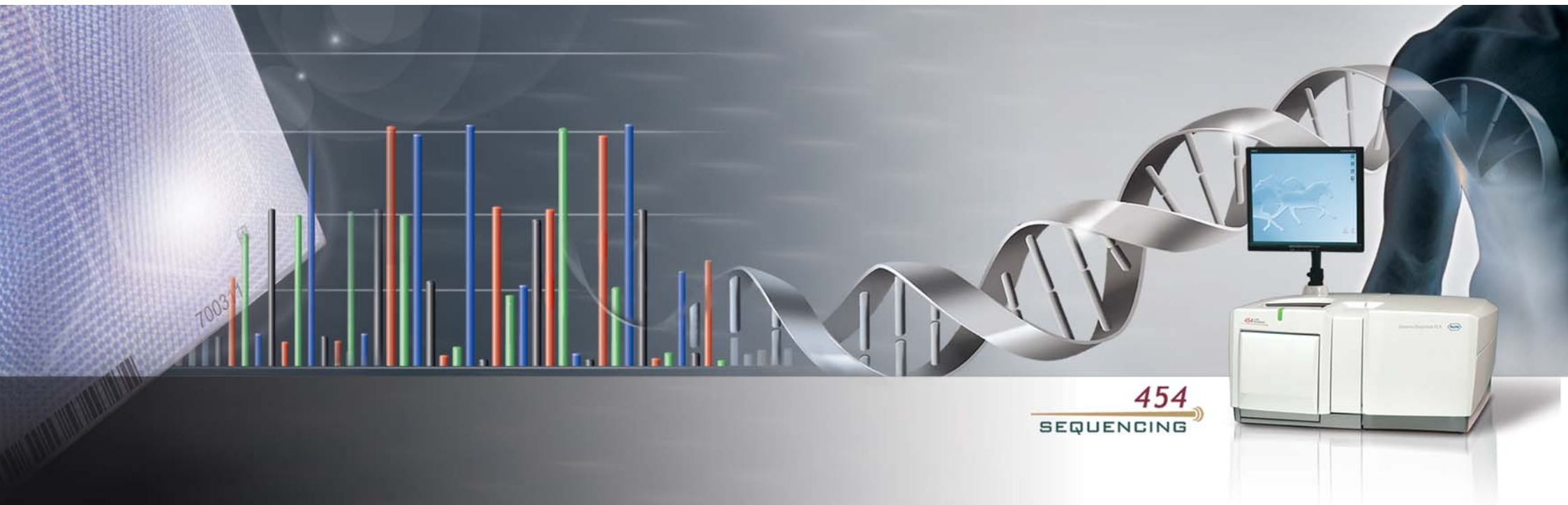


Sequencing Method Manual

GS FLX Titanium Series


October 2009 (Rev. November 2010)

For life science research only. Not for use in diagnostic procedures.



454
SEQUENCING

1. Workflow

 This procedure can also be used to perform a test sequencing Run, using only Control DNA Beads. This requires a GS FLX Titanium Control Bead Kit, in addition to all the materials used for a standard Run. The only difference is the preparation of the DNA Beads. For details of the preparation of the Control DNA Beads, please see the Package Insert to the GS FLX Titanium Control Bead Kit.

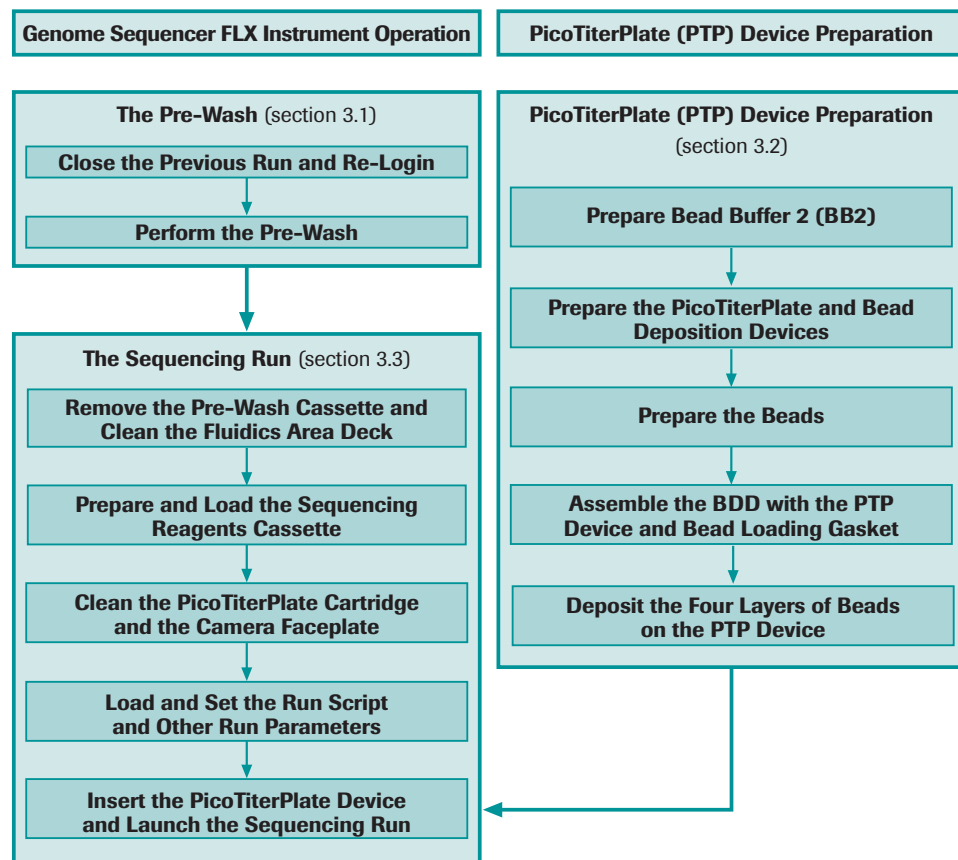


Figure 1: Workflow of a sequencing Run performed on the Genome Sequencer FLX Instrument

Note that instrument preparation and PicoTiterPlate device preparation are done in parallel such that the sequencing Run is initiated as soon as the PicoTiterPlate device is ready.

2. Before You Begin

2.1 What You Should Have Before Starting

2.1.1 Sample

The sample library being sequenced must have been prepared using the GS FLX Titanium methods and kits. The library will then have been amplified using one of the GS FLX Titanium emPCR Kits.

2.1.2 Required Genome Sequencer FLX System Equipment and Reagents

Refer to the customer-restricted 454 Web page at www.454.com/my454 for the lists of nonconsumables and material required but not provided.

2.1.3 GS Sequencing Kit

The GS FLX Titanium Sequencing Kit XLR70 is used in combination with the matching GS FLX Titanium PicoTiterPlate Kit 70 × 75. These kits provide reagents and components necessary for a single sequencing Run. Refer to the customer-restricted 454 Web page at www.454.com/my454 for a complete list of kits and reagents.

2.1.4 Choosing the Size and Number of PTP Regions for Your Sample

The GS FLX Titanium Sequencing and PicoTiterPlate Kits support various bead loading region configurations. The size and number of PTP regions depend on the sequencing throughput requirements of the experience, in total bases or total reads. To determine the proper gasket size, proceed as follows:

Use Table 1 to identify the size and number of bead loading regions best suited to yield the sequencing throughput for your DNA library sample. The Mbp throughput presented here is typical for good “Extra Long Read”, 200 cycles, sequencing Runs performed a good quality shotgun libraries. For example, column 5 shows the expected throughput of a full sequencing Run carried out with the longest run script available (200 cycles, generating an average read length of 350 to 450 nt), with all regions loaded.

Region Size	PTP Device Size	Regions per PTP Device	Bases/Region (Mbp)	Bases per Full PTP Device (Mbp)	Reads/Region (× 10 ³)
Large	70 × 75 mm	2	180 – 280	360 – 560	450 – 650
Medium	70 × 75 mm	4	60 – 110	240 – 440	160 – 250
Medium/Small	70 × 75 mm	8	30 – 55	240 – 440	80 – 120
Small	70 × 75 mm	16	10 – 20	160 – 320	25 – 40

Table 1: Gasket specification and throughput

Sequencing Method Manual

Use the following examples as guidance of gasket loading for your experiment:

- ▶ If the experiment aims to sequence 10 Mbp at 20-fold coverage, a total throughput of 200 Mbp is required. This can be obtained in a single large region of a PTP device.
- ▶ Sequencing 80 Mbp at a 20-fold coverage, requires 1.6 Gbp of total throughput and about 8 large regions, or 4 full sequencing Runs with the large region gasket.
- ▶ Resequencing 4 Mbp at 8-fold coverage yields a total 32 Mbp throughput and requires one single medium-small region.

! Library Samples Type on PTP Device: Libraries with different sequencing keys cannot be loaded in the same region of a PTP device. The PTP device can hold, in different regions, libraries with different sequencing keys.

Two sequencing keys exist for libraries:

- ▶ TCAG for General, Paired End, and Amplicon libraries
- ▶ GACT for Rapid and cDNA Rapid libraries

2.1.5 Reagents and Titanium Bead Buffer

The Sequencing Reagents XLR70 of the GS FLX Titanium Sequencing Kit XLR70 must be thawed prior to use in the sequencing procedure described in this manual. Also, the bottle of Titanium Bead Buffer must be pre-chilled because it will be supplemented with apyrase, a thermolabile enzyme, to make Bead Buffer 2.

Before beginning an experiment, do the following:

- 1 Bring the Sequencing Reagents tray of the GS FLX Titanium Sequencing Kit XLR70 out of its frozen storage. Keep the Sequencing Enzymes sleeve at -15 to -25°C.
- 2 Remove the bag from the Sequencing Reagents tray, and allow the reagents to thaw, using one of the following procedures, keeping the tray upright and protected from bright light.
 - ▶ Immerse (do not allow the water level to reach the tube caps) in a room temperature (+15 to +25°C) water bath for 2 h.
 - ▶ Leave for 2.5 h at room temperature (+15 to +25°C).
 - ▶ Leave at +2 to +8°C for up to 60 h (e.g. weekend).
- 3 Place the bottle of Titanium Bead Buffer on ice.
- 4 When the contents of the Sequencing Reagents tray are thawed, transfer the tray to +2 to +8°C to keep the reagents chilled until the Run. Do not let the reagents remain at this temperature for more than 48 h.
- 5 Within 3 h of beginning the procedure, place the reagents of the Sequencing Enzyme sleeve to thaw on ice. Do not re-freeze the reagents.

3. Procedure

3.1 The Pre-Wash



Under normal conditions of continuous operation, the instrument is kept running after a Run completed. Therefore, the PTP device from the previous Run should still be in place in the instrument's PTP cartridge, within the camera door. If there is no PTP device on the cartridge, you must install a used but intact one (and a cartridge seal) before proceeding with the pre-wash.

- 1 Close the previous run by clicking **OK** on the "Sequencing run complete" window.
- 2 If required, log in as follows:
 - ▶ Click on "Operator" in the Status area of the application window, Figure 2.



Figure 2: The upper login window of the GS Sequencer application

- ▶ Login by selecting your user name and click "Sign In". The main window of the GS Sequencer application will open (Figure 3).

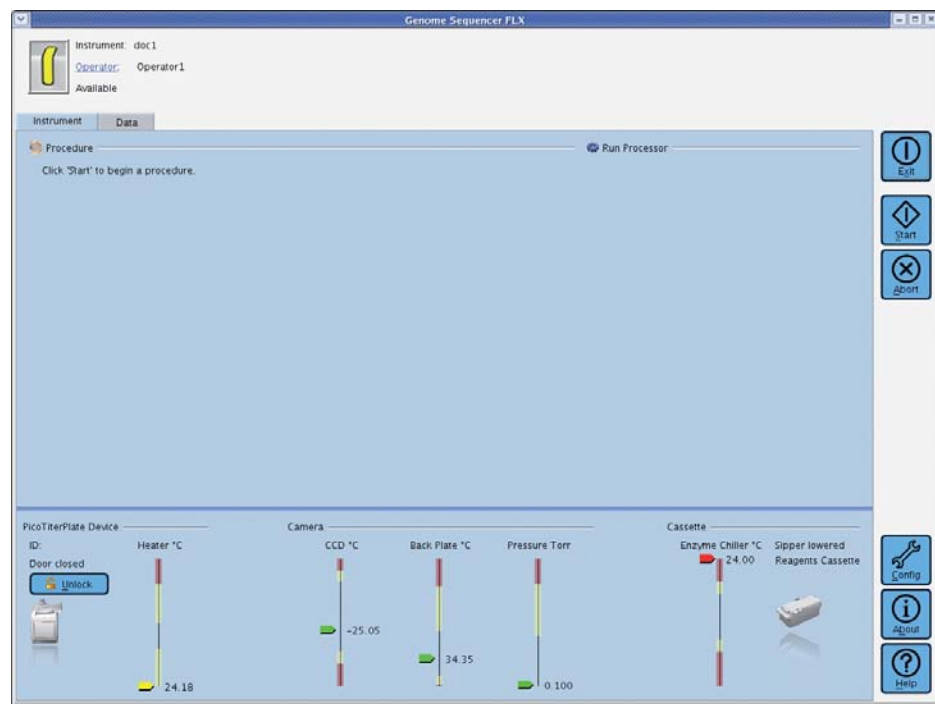


Figure 3: The GS Sequencer application window after an Operator logs in

- 3 Perform a pre-wash by discarding the spent reagents and cleaning the Reagents cassette:
 - ▶ Open the exterior fluidics door and raise the sipper manifold completely (Figure 4A).
 - ▶ Slide the Reagents cassette out (Figure 4B).

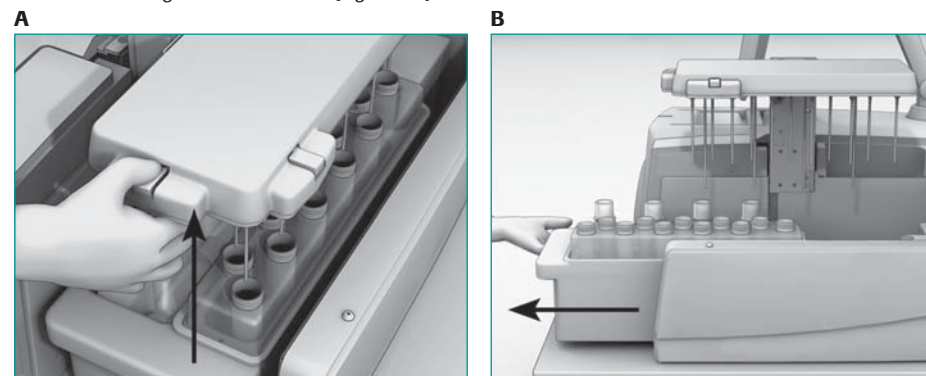


Figure 4: Removing the Reagents cassette (from the previous Run)

- ▶ Pour out the fluid remaining in the container and trash the spent bottles and tubes.
- ▶ Thoroughly rinse the empty Reagent cassette with warm tap water.
- ▶ Dry all the outside surfaces of the cassette with a paper towel.
- ▶ Remove and discard used Sipper Tubes, and change gloves.
- ▶ Replace the Sipper Tubes, and change gloves to avoid contaminating other components.
- ▶ Prepare the pre-wash cassette by placing the GS FLX Pre-wash Tube Holder on top of the Reagents cassette and by inserting 11 small tubes and 4 large tubes in the cassette. Fill all the tubes with Pre-wash Buffer and slide the cassette in the instrument, lower the manifold and close the fluidics door (Figure 5).

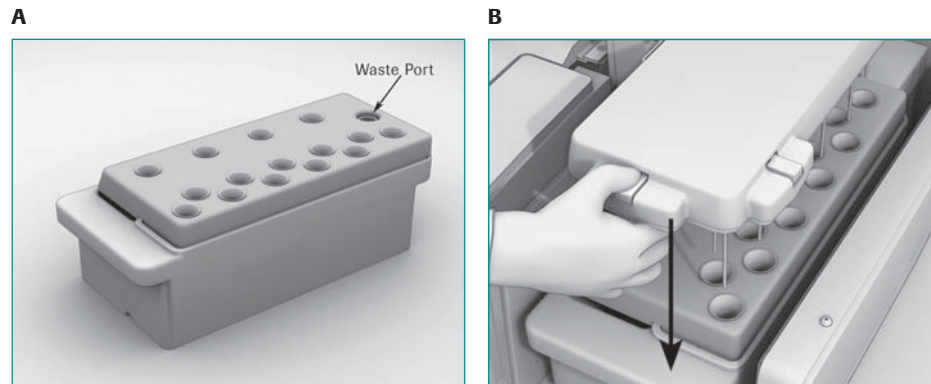


Figure 5: The Pre-wash cassette; loading before a pre-wash

- 4 If necessary, replace bent Sipper Tubes, as described in the *Genome Sequencer FLX Instrument Owner's Manual*.
- 5 If there isn't a PTP device or if the cartridge seal is leaking, install a used but intact PTP device (with a used but intact cartridge seal) following instructions in Section 3.3.5.

3.1.1 Launch the Pre-Wash

- 1 Return to the instrument computer. If the Instrument tab is not selected, select it now (Figure 6).



Figure 6: The Instrument tab of the GS Sequencer software

- 2 Click the Start button in the Global action area.
 - ▶ This opens the Run Wizard's first window: Choose a procedure (Figure 7).

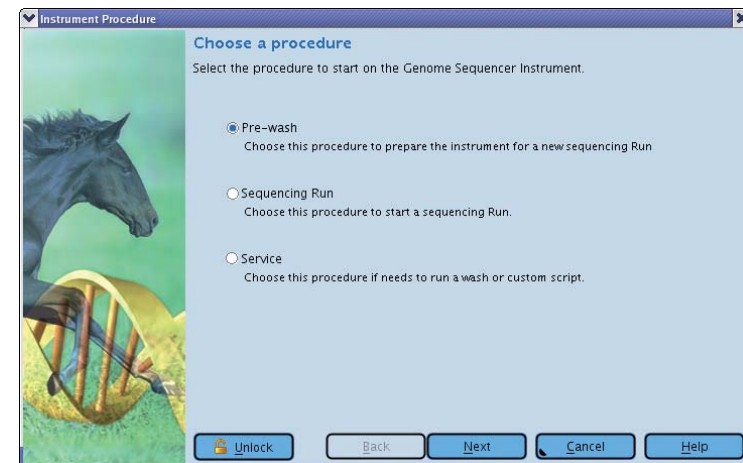


Figure 7: The Run Wizard's first window (Choose a procedure), with the Pre-wash option selected

- In the Run Wizard's first window, select the Pre-wash option, and click the Next button.

► This opens the Run Wizard's second window: Start Pre-wash (Figure 8).

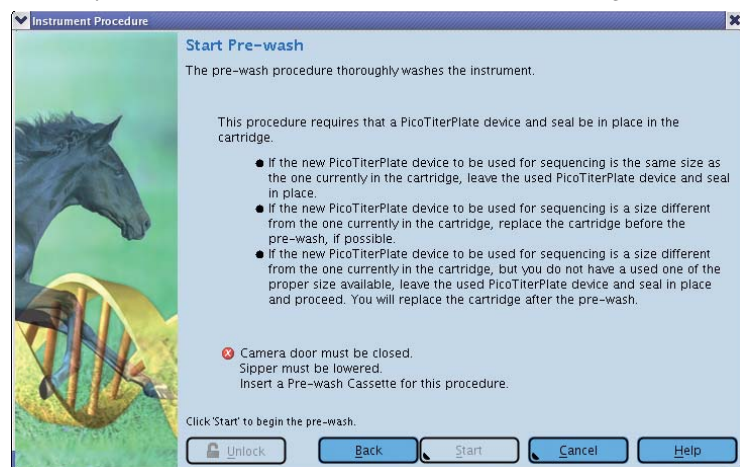


Figure 8: The Run Wizard's second window (last Wizard window for Pre-wash): Start Pre-wash

- Click the Start button in the Start Pre-wash window (from the Run Wizard) to start the Pre-wash operation.

Monitor the instrument until the Status LED located above the camera door on the instrument is blinking green. If the instrument encounters any problems during the initiation of the Pre-wash, a message describing the issue will appear in the Status area of the GS Sequencer window.

PicoTiterPlate device preparation: Start the preparation of the PTP device (Section 3.2) as soon as the Pre-wash is safely ongoing. The Pre-wash will proceed to completion without any further user intervention (approximately 1 hour).

There is an *Abort* button available in the GS Sequencer software main window (Figure 6), which can be clicked if a problem occurs.

3.2 PicoTiterPlate (PTP) Device Preparation

3.2.1 Prepare Bead Buffer 2 (BB2)

- Add **1.2 ml** of Titanium Supplement CB to the **200 ml** bottle of pre-chilled Titanium Bead Buffer. Re-cap and mix gently by inversion.
- Remove the tube of Apyrase from the Sequencing Enzymes sleeve and completely thaw it if necessary. Leave the other components on ice until needed.
- Add **34 µl** of Apyrase solution to the bottle of Titanium Bead Buffer plus Titanium Supplement CB.
- Re-cap and label the bottle "Bead Buffer 2". Mix gently by inversion, and return the bottle to ice. Also return the remainder of the Titanium Supplement CB and of the apyrase to ice.

3.2.2 Prepare the PicoTiterPlate and Bead Deposition Devices

- Retrieve the PTP device shipping tray. Lift the PTP device out of the shipping tray with a gloved hand and place it back into the tray.
- Note down the PTP device ID to enter the 6-digit number in the second window of the Run Wizard (Section 3.3.4).
- Completely submerge the PTP device in BB2 for at least 10 minutes at room temperature until you are ready to assemble the Bead Deposition Device. Keep the rest of the BB2 on ice.
- Wash the bead loading gasket, by gently shaking the gasket and seal for 30 seconds in a Sparkleen solution (or nanopure water if Sparkleen is not available). Rinse thoroughly with nanopure water and let air dry on a paper towel.
- Wash the appropriate BDD using a soft bristle brush and Sparkleen solution. Rinse thoroughly with nanopure water and let the device air dry on a paper towel.

3.2.3 Prepare the Beads

The Genome Sequencer FLX System contains 4 kinds of microparticles (beads), as listed in Table 2. Each type of bead must undergo a specific preparation procedure, as described below.

Bead Type	Description / Function
DNA Beads	DNA Beads carry the DNA library to be sequenced (sample). These sample DNA Beads must have been prepared using the appropriate Genome Sequencer System procedures and kits. Before use, the sample DNA Beads will be spiked (3.2.3.2) with Control DNA Beads (included in the GS FLX Titanium Sequencing Kits), which serve as an internal control for the sequencing reaction. Control DNA Beads are also available in a GS FLX Titanium Control Bead Kit, which can be used to carry out a separate, test sequencing Run (see the Pack Insert to the GS FLX Titanium Control Bead Kit).
Enzyme Beads	Enzyme Beads carry the immobilized enzyme components of the chemiluminescence system (sulfurylase and luciferase).
PPiase Beads	PPiase Beads scavenge inorganic pyrophosphate (PPi) to reduce well-to-well crosstalk and interference during each nucleotide flow, as well as residual background noise after and between flows.
Packing Beads	Packing Beads stabilize and maintain all the immobilized components of the system within the wells of the PTP device, throughout the sequencing Run.

Table 2: The 4 types of beads used in the GS FLX Titanium System chemistry

3.2.3.1 Prepare the Packing Beads

- 1 Wash the Packing Beads **three times** in BB2 with **1 ml** of BB2 by centrifuging at **10,000 rpm** ($9300 \times g$ RCF) for **5 minutes**. Break up aggregates by vortexing, until a uniform suspension is achieved.
 - ▶ You may start preparing the DNA Beads during these three centrifugations.
- 2 After the third wash, add **550 μ l** of BB2 per tube and re-suspend the beads and keep on ice.

3.2.3.2 Prepare the DNA Beads (Sample and Control)

- 1 Using Table 3, determine the number of sample DNA beads required for your gasket (column 3).
- 2 Calculate the volume of DNA library beads to be sequenced based on the number of beads needed and the concentration of the library, in beads/ μ l.
- 3 Vortex the DNA library beads and transfer the appropriate amount of beads into clean tubes of the appropriate size (*e.g.* 2 ml tubes for large regions, 1.7 ml tubes for medium or M/S regions, and 0.2 ml tubes for small regions). **Use a separate tube for each loading region.**
- 4 Add the appropriate volume of Control DNA Beads suspension to each DNA library bead tube (column 4).
- 5 Spin the beads for **1 minute** at 10,000 rpm. Rotate the tube 180° and spin again for **1 minute**.
 - ▶ If the total volume of bead suspension is equal to or less than the volume indicated in column 5, skip this centrifugation, as you have no volume to remove.
- 6 Calculate the volume of supernatant to remove that will leave the volume indicated in column 5, for the bead loading gasket configuration you are using. This volume will vary depending on the volume of DNA library beads used, which in turn depends on the concentration of the DNA library.
- 7 Set a pipettor to the volume calculated in step 6, above. Being careful not to disturb the bead pellet, carefully and slowly remove the appropriate amount of supernatant and discard it.

Loading Region Size	PicoTiterPlate Size	Number of DNA Library Beads to Load per Region	Volume of Control DNA Beads (μ l)	Target Final Volume, after Centrifugation (μ l)
Large	70 \times 75 mm	2,000,000 (\times 2)	20 (\times 2)	50 (\times 2)
Medium	70 \times 75 mm	790,000 (\times 4)	10 (\times 4)	30 (\times 4)
Medium/Small	70 \times 75 mm	340,000 (\times 8)	6 (\times 8)	15 (\times 8)
Small	70 \times 75 mm	125,000 (\times 16)	3 (\times 16)	10 (\times 16)

Table 3: Preparation of the DNA Beads, for each bead loading gasket configuration



- 8 Separately, prepare the **DNA Bead Incubation Mix (DBIM)** in a clean 15 ml tube (Table 4). Vortex gently to ensure that the Polymerase Cofactor and DNA Polymerase are thoroughly mixed.
- ▶ Bulk preparation of DBIM is fine, even for gaskets with multiple loading regions. The proper amount will be pipetted into individual tubes of DNA beads in the next step.

Loading Region Size	PicoTiterPlate Size	BB2 (μl)	Polymerase Cofactor (μl)	DNA Polymerase (μl)	Total Volume (μl)
All sizes	70 × 75 mm	1570	150	300	2020

Table 4: Preparation of the DNA Bead Incubation Mix

- 9 Add the DBIM to each tube of DNA beads, per Table 5. Vortex well.
- ▶ If the volume of DNA beads in your tubes is less than the volume indicated in the third column, add extra DBIM so each tube contains the **Total Volume** indicated in the last column.
 - ▶ The leftover DBIM can be discarded.

Loading Region Size	PicoTiterPlate Size	DNA Beads (μl)	DBIM (μl)	Total Volume (μl)
Large	70 × 75 mm	50 (× 2)	950 (× 2)	1000 (× 2)
Medium	70 × 75 mm	30 (× 4)	320 (× 4)	350 (× 4)
Medium/Small	70 × 75 mm	15 (× 8)	140 (× 8)	155 (× 8)
Small	70 × 75 mm	10 (× 16)	50 (× 16)	60 (× 16)

Table 5: Dilution of DNA Beads in DNA Bead Incubation Mix

- 10 Incubate the samples on the lab rotator at room temperature for **15 minutes**.
- ▶ It is convenient to prepare the beads of layers 1, 3, and 4 during this 15 min of incubation.
 - ▶ Do not exceed 50 min for this incubation.

3.2.3.3 Prepare the Enzyme and PPIase Beads (Bead Layers 1, 3 & 4)

These two types of beads can be washed in parallel, in separate tubes. Make sure to change pipette tips to avoid contaminating them with one another.

- 1 Vortex and pellet the Enzyme Beads and the PPIase Beads using a Magnetic Particle Concentrator (MPC) for **30 seconds** for the beads to pellet. Invert the MPC several times and wait **30 seconds** again. Carefully remove the supernatants and remove the tubes from the MPC.
- 2 Wash the Enzyme and PPIase Beads **three times** with **1 ml** of BB2 for each tube of Enzyme Beads and with **500 μl** of BB2 for the tube of PPIase Beads. Vortex and wash the beads using the MPC, as above.
- 3 After the third wash, add **1 ml** of BB2 to each tube of Enzyme Beads and **500 μl** of BB2 to the tube of PPIase Beads, and vortex the pellets. Keep on ice.
- 4 Prepare the beads for layers 1, 3, and 4 in 3 separate appropriately labeled tubes of an appropriate size (e.g. 15 ml). This is done by diluting the Enzyme Beads (layer 1: Enzyme Beads pre-layer; and layer 3: Enzyme Beads post-layer) and the PPIase Beads (layer 4) in BB2, as listed in Table 6. Vortex the washed Enzyme and PPIase Beads to get a uniform suspension before transferring.

Bead Layer	Kit	BB2 (μl)	Enzyme Beads (μl)	PPIase Beads (μl)	Total Volume (μl)
Layer 1	XLR70	3250	550	---	3800
Layer 3		2500	1300	---	3800
Layer 4		3340	---	460	3800

Table 6: Dilution of the Enzyme and PPIase Beads for bead layers 1, 3, and 4

3.2.3.4 Combine the DNA and Packing Beads (Bead Layer 2)

- 1 After the DNA beads incubation in DBIM is complete, return to the washed Packing Beads and the leftover BB2. Vortex and transfer the appropriate volumes of washed Packing Beads and of BB2 to the tubes containing the DNA beads, as listed in Table 7.

Loading Region Size	PicoTiterPlate Size	DNA Beads (μl)	Packing Beads (μl)	BB2 (μl)	Total Volume (μl)
Large	70 × 75 mm	1000 (× 2)	265 (× 2)	435 (× 2)	1700 (× 2)
Medium	70 × 75 mm	350 (× 4)	100 (× 4)	210 (× 4)	660 (× 4)
Medium/Small	70 × 75 mm	155 (× 8)	45 (× 8)	125 (× 8)	325 (× 8)
Small	70 × 75 mm	60 (× 16)	15 (× 16)	35 (× 16)	110 (× 16)

Table 7: Preparation of bead layer 2

- 2 Vortex and rotate the DNA and Packing Beads mixes **at least 5 min** at room temperature. The unused packing beads can be discarded.

Sequencing Method Manual

3.2.4 Assemble the BDD with the PTP Device and Bead Loading Gasket

- 1 Using a gloved hand, remove the PTP device from the BB2 bath, handling it by the edges only and pour off excess BB2.
- 2 Wipe the back of the PTP device with a Kimwipe, so that it is completely dry. However, be careful NOT to allow the side with the wells to run dry.
- 3 Place the PTP device onto the BDD base (Figure 9A), aligning the notched corners of the PTP device and the BDD base.
- 4 Secure the washed and dried bead loading gasket to the BDD base by laying it on top of the PTP device and placing its molded ridge into the groove on the BDD base (Figure 9B). Align the notched corners of the bead loading gasket and the BDD base, as shown in the Figure.
 - ! **16 “Small” Regions Bead Loading Gasket:**
 - ▶ The region separators of this gasket sometimes stick together. If this happens, gently separate them manually before laying the gasket on top of the PTP device, in the BDD.
 - ▶ After installing this gasket in the BDD, gently spread it lengthwise to ensure that the lanes are straight and uniform.
- 5 Carefully place the BDD top over the assembled BDD base/PTP device/Gasket (Figure 9C) so the port holes and air vents line up with the loading regions defined by the gasket, underneath. Align the dowels on the BDD base so they slide into the holes in the BDD top and the BDD top is sitting flat across the PTP device.
- 6 Press down on the top of the BDD, and rotate the two latches from the BDD base into the grooves in the BDD top to firmly secure the assembly (Figure 9D). When you hear a ‘click’, the latches should be firmly seated in the grooves, providing the correct amount of pressure to maintain a liquid-tight seal.

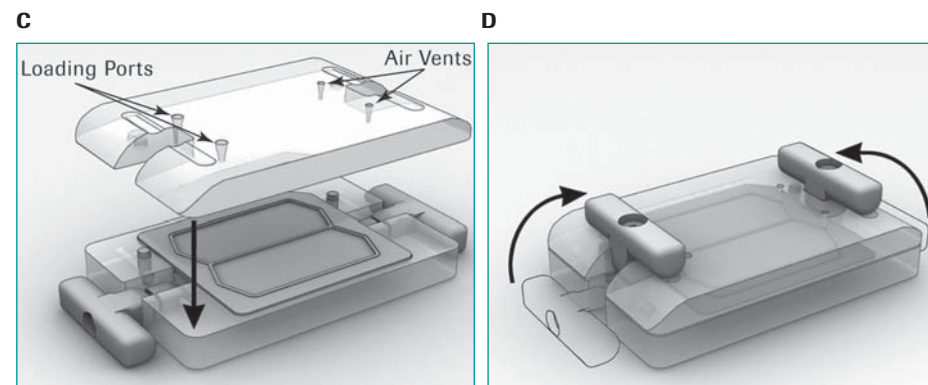
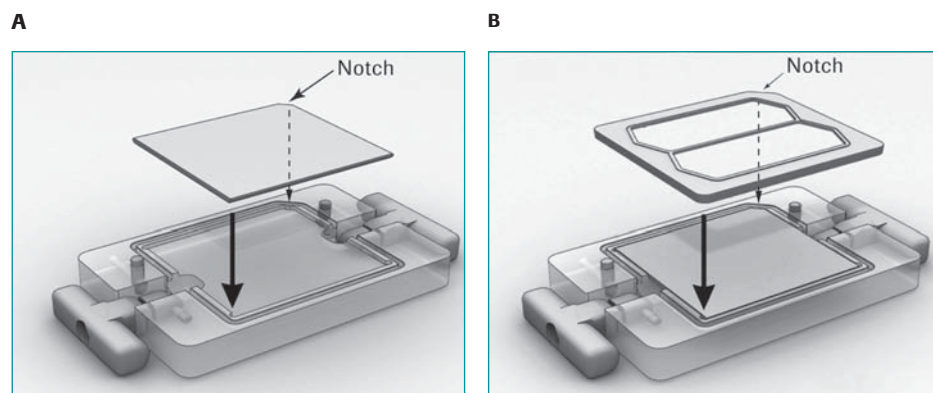


Figure 9: Assembly of the Bead Deposition Device

3.2.5 Deposit the Four Layers of Beads on the PTP Device

Deposit the beads onto the PTP device by injecting the bead suspension through the port holes of the assembled Bead Deposition Device (BDD), and then using centrifugal sedimentation to settle the beads to the bottom of the PicoTiterPlate wells. This process is repeated for each of four layers:

- ▶ Bead layer 1: Enzyme Beads pre-layer
 - ▶ Bead layer 2: DNA and Packing Beads layer
 - ▶ Bead layer 3: Enzyme Beads post-layer
 - ▶ Bead layer 4: PPIase Bead layer
- ! **Pipette tips:** Use the correct pipette tips to load beads: 2 ml tip for Large regions, 1 ml tip for Medium and M/S regions, and 200 μ l tip for Small regions.
 - ▶ **Time between centrifugations:** Minimize the time interval between loading the beads and starting the centrifugation.
 - ! **Bead distribution:** Load the beads promptly for an even distribution in the PTP device.
 - ▶ **Air bubbles:** Avoid injecting air into the BDD.
 - ▶ **Bead delivery:** Use a single, even injection to fill each loading region of the BDD.
 - ▶ **Loading region fill:** Fill the BDD completely but do not overflow the loading regions. Discard any excess bead mix.

3.2.5.1 Deposit Bead Layer 1: the Enzyme Beads Pre-Layer

- 1 Vortex the bead suspension for layer 1 for 5 seconds to obtain a homogeneous suspension.
- 2 Using a pipette and tip of the proper size, draw the appropriate amount of bead suspension for the loading region size you are using, per Table 8.

Loading Region Size	PicoTiterPlate Size	Volume to Load (µl)
Large	70 × 75 mm	1860 (× 2)
Medium	70 × 75 mm	660 (× 4)
Medium/Small	70 × 75 mm	325 (× 8)
Small	70 × 75 mm	110 (× 16)

Table 8: Volume of bead layer 1 (Enzyme Beads pre-layer) to load per region for each bead loading gasket configuration. M/S: Medium/Small

- 3 Completely resuspend the beads (avoid creating bubbles) and promptly load the bead suspension onto the first region of the PTP device, through the port hole on the BDD top (see Figure 10). Make sure to use a single, smooth dispensing action to ensure even distribution of the beads over the entire region of the PTP device.

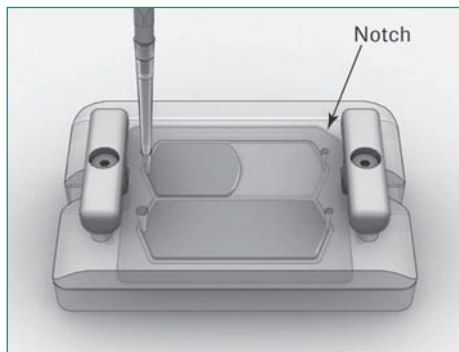


Figure 10: Filling the loading regions of the assembled Bead Deposition Device

- 4 Repeat steps 1 to 3 for all loading regions of the PTP device.
- 5 **For large region Bead Deposition Devices only:** Cover the loading ports and the vent holes with the BDD port seals provided in the GS FLX Titanium PicoTiterPlate Kit 70 × 75.
- 6 Place both the assembled BDD and the BDD counterweight into centrifuge swinging baskets as appropriate for your centrifuge rotor, and place them onto the rotor opposite each other.
- 7 Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 × g** (2640 RPM for the Beckman Coulter X-12 or X-15 centrifuges).

! Step synchronization: Perform the steps of Section 3.3.1 during this 5 minutes centrifugation.

3.2.5.2 Deposit Bead Layer 2: the DNA and Packing Beads

- 1 Remove the BDD from the centrifuge, and discard the BDD port seals that cover the loading ports and air vents.
- 2 With a pipettor, gently remove as much of the supernatants as possible through the port holes on the BDD top.
- 3 Remove the tubes of bead suspension for layer 2 from the rotator.
- 4 Briefly spin the bead suspension for layer 2 to collect material from the cap. Pipette up and down to mix.
- 5 Using a pipette and tip of the proper size, draw the appropriate amount bead suspension for the loading region size you are using, per Table 9.

Loading Region Size	PicoTiterPlate Size	Volume to Load (µl)
Large	70 × 75 mm	1700 (× 2)
Medium	70 × 75 mm	660 (× 4)
Medium/Small	70 × 75 mm	325 (× 8)
Small	70 × 75 mm	110 (× 16)

Table 9: Volume of bead layer 2 to load per region for each bead loading gasket configuration

- 6 Very carefully, pipette up and down three times to resuspend the beads completely (avoid spilling or creating bubbles) and promptly load them onto the first region of the PTP device, through the port hole on the BDD top, (see Figure 10).
- 7 Repeat steps 4 to 6 for all loading regions of the PTP device.
- 8 Cover the loading ports and the vent holes with the BDD port seals provided in the GS FLX Titanium PicoTiterPlate Kit 70 × 75.
- 9 Centrifuge the loaded PTP device in the BDD for 10 minutes at **1620 × g** RCF (2640 rpm for the X-12 or the X-15 centrifuges), as above.

! Step synchronization: Perform the steps of Section 3.3.2 during this 10 minutes of centrifugation.


Sequencing Method Manual


3.2.5.3 Deposit Bead Layer 3: the Enzyme Beads Post-Layer

- 1 Change gloves after loading the Reagents cassette into the instrument.
- 2 Remove the BDD from the centrifuge, and discard the BDD port seals that cover the loading ports and air vents.
- 3 With a pipettor, gently draw out all the supernatant from the centrifuged bead layer 2, through the port holes on the BDD top.
 - ▶ It is useful to save this bead layer 2 supernatants, for troubleshooting purposes in case the sequencing Run does not produce the expected number of reads.
- 4 Vortex the bead suspension for layer 3 for 5 seconds to obtain a uniform suspension.
- 5 Using a pipette and tip of the proper size, draw the appropriate amount of bead suspension for the loading regions you are using, per Table 10.

Loading Region Size	PicoTiterPlate Size	Volume to Load (µl)
Large	70 × 75 mm	1860 (× 2)
Medium	70 × 75 mm	660 (× 4)
Medium/Small	70 × 75 mm	325 (× 8)
Small	70 × 75 mm	110 (× 16)

Table 10: Volume of bead layer 3 (Enzyme Beads post-layer) to load per region for each bead loading gasket configuration

-  For the third and fourth layers, these volumes may be more than needed to fill the loading regions, due to remaining fluid volume leftover on the PTP device. This is not a problem.
- 6 Pipette up and down three times to resuspend the beads completely (avoid creating bubbles) and promptly load the bead suspension onto the first region of the PTP device, through the port hole on the BDD top (see Figure 8).
- 7 Repeat steps 4 to 6 for all loading regions of the PTP device.
- 8 Cover the loading ports and the vent holes with new BDD port seals provided in the GS FLX Titanium PicoTiterPlate Kit 70 × 75.
- 9 Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 × g** RCF (2640 rpm for the Beckman Coulter X-12 or X-15 centrifuges), as above.

 **Step synchronization:** Perform the steps of Sections 3.3.3 and 3.3.4 during this 5 minutes centrifugation.


3.2.5.4 Deposit Bead Layer 4: the PPIase Beads

- 1 Remove the BDD from the centrifuge, and discard the BDD port seals that cover the loading ports and air vents.
- 2 With a pipettor, gently draw out and discard all the supernatant from the centrifuged bead layer 3, through the port holes on the BDD top.
- 3 Vortex the bead suspension for layer 4 for 5 seconds to obtain a uniform suspension.
- 4 Using a pipette and tip of the proper size, draw the appropriate amount of bead suspension for the loading regions you are using, per Table 11.

Loading Region Size	PicoTiterPlate Size	Volume to Load (µl)
Large	70 × 75 mm	1860 (× 2)
Medium	70 × 75 mm	660 (× 4)
Medium/Small	70 × 75 mm	325 (× 8)
Small	70 × 75 mm	110 (× 16)

Table 11: Volume of bead layer 4 (PPIase Beads) to load per region for each bead loading gasket configuration

- 5 Pipette up and down three times to resuspend the beads completely (avoid creating bubbles) and promptly load the bead suspension onto the first region of the PTP device, through the port hole on the BDD top (see Figure 10).
- 6 Repeat steps 3 to 5 for all loading regions of the PTP device.
- 7 Cover the loading ports and the vent holes with new BDD port seals provided in the GS FLX Titanium PicoTiterPlate Kit 70 × 75.
- 8 Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 × g** RCF (2640 rpm for the Beckman Coulter X-12 or X-15 centrifuges), as above.

 **Step synchronization:** By the time you reach this point in the procedure, you should have already performed the steps of Sections 3.3.1 through 3.3.4. If you have not, complete these steps now. If you have, proceed with Section 3.3.5. You will collect the fully prepared PTP device from the centrifuge in step 3 of that section.

3.3 The Sequencing Run

3.3.1 Remove the Pre-Wash Cassette and Clean the Fluidics Area Deck

! **Step synchronization:** Perform the procedures described in this section during the 5 minutes centrifugation of the bead layer 1.

- 1 On the instrument computer, a Sequencing run complete message will be displayed in the Status area of the GS Sequencer software main window. Click **OK** to continue.
- 2 Open the exterior fluidics door and raise the sipper manifold completely (see Figure 11A).
- 3 Slide out the Pre-wash Reagents cassette (Figure 11B).

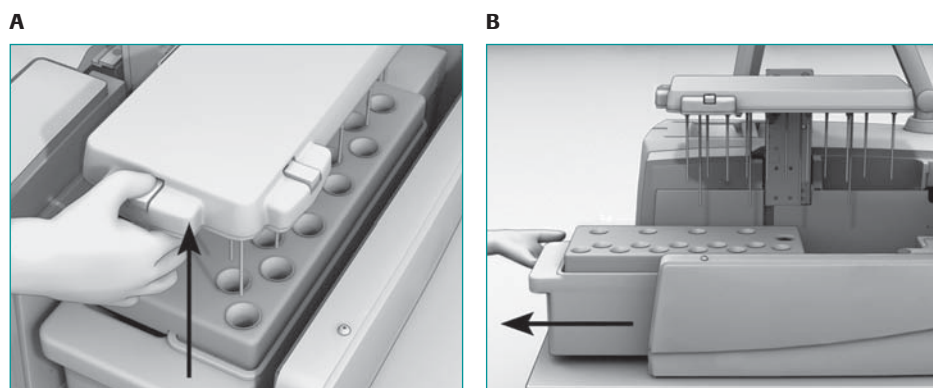


Figure 11: Removing the Pre-wash cassette from the instrument

- 4 Remove the Pre-wash Tubes and the GS FLX Pre-wash Tube Holder from the Reagents cassette. Empty the tubes in a sink and discard them.
 - !** The Pre-wash Tubes should be discarded after every use. Never re-use Pre-wash Tubes.
- 5 Wipe all the outside surfaces of the Reagents cassette with a paper towel.
- 6 Wipe the fluidics area deck (inside the instrument) with 50% ethanol and a paper towel, being careful to not touch the Sipper Tubes. Allow the deck to air dry completely.

! **Sipper Tubes contamination risk:** Never touch the Sipper Tubes unless you are replacing them. If contamination of any of the lines is suspected, replace them by following the instructions provided in the *Genome Sequencer FLX Owner's Manual*.

! **Step synchronization:** Return to the bead deposition procedure for the DNA and Packing Beads layer (bead layer 2, Section 3.2.5.2).

3.3.2 Prepare and Load the Sequencing Reagents Cassette

- !** **Step synchronization:** Perform the procedures described in this section during the 10 minutes centrifugation of the DNA and Packing Beads layer (bead layer 2).
- ▶ Chilled reagents:** The concentrated reagents of the Sequencing Reagents tray **MUST** be thawed but kept refrigerated (+2 to +8°C).

! **Sodium Chlorite tablet:** The GS FLX Titanium Sequencing system requires a sodium chlorite tablet. Sodium chlorite is a Hazard to humans and domestic animals.

- ▶ DANGER: Corrosive.** Causes irreversible eye damage & skin burns. Harmful if absorbed through skin. Harmful if swallowed. Do not get in eyes, on skin or on clothing. Wear appropriate protective eyewear (safety glasses), clothing and gloves. Wash thoroughly with soap and water after handling. Remove contaminated clothing & wash clothing before use.
- ▶ Environmental Hazard:** This product is toxic to fish and other aquatic organisms.
- ▶ Physical/Chemical Hazards:** Keep away from combustible, organic or other readily oxidizable materials.
- ▶ Storage and Disposal:** Store in cool, dry area away from sunlight and heat. Place empty foil pouch in trash.
- ▶ If sodium chlorite tablets are not available:** For countries where sodium chlorite tablets are not available, proceed without it. Then, perform a separate Maintenance Wash Run after every two Sequencing Runs, to keep your system clean and help prevent the potential build-up of foreign materials in the fluidics lines.

- 1 Add **6.6 ml** of Titanium Supplement CB and **1000 µl** of DTT to **each** bottle of Titanium Buffer CB. Re-cap and gently invert the bottles to mix.
- 2 Place the Sequencing Reagents tray, kept in the cold, in the right-hand side of the Reagents cassette.
- 3 Dilute **5 µl** of the PPIase reagent in **45 µl** Inhibitor TW reagent in a 1.7 ml tube, and vortex.
- 4 Supplement the 4 reagents from the Sequencing Reagents tray with their appropriate additives, as listed in Table 12. Add the reagent supplements to one tube at a time, in the order they appear in the table, following instructions in the Notes. Once all the reagents are added, change gloves.

Kit	Sodium Chlorite tablet to add to the tube of "Post Run Wash"	Volume of Diluted PPIase to add to "Inhibitor TW"	Volume of Apyrase to add to "Buffer for Apyrase"	Volume of dATP to add to "Buffer for dATP (A)"
XLR70	1 tablet	13.2 µl	260 µl	3000 µl
Notes	<ul style="list-style-type: none"> ▶ Tray position 1 ▶ White sticker ▶ Let fizz 2 minutes ▶ Change gloves 	<ul style="list-style-type: none"> ▶ Used diluted PPIase ▶ Tray position 9 ▶ Pink sticker 	<ul style="list-style-type: none"> ▶ Tray position 11 ▶ Yellow sticker ▶ Change gloves 	<ul style="list-style-type: none"> ▶ Tray position 10 ▶ Lavender sticker ▶ Change gloves

Table 12: Supplementing Sequencing Reagents with Sodium Chlorite, PPIase, Apyrase, and dATP

Sequencing Method Manual

- Invert the tray 20 times to uniformly mix the contents of all tubes. Make sure that there are no undissolved particulates in any of the reagents (pay particular attention to the tube of Substrate reagent), and place the tray back in the Reagents cassette with the lone Apyrase tube at the far end, as shown in Figure 12, below.

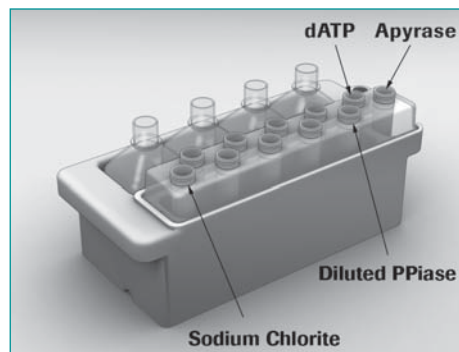


Figure 12: Loading the Reagents cassette, with the Sequencing Reagents tray in the proper orientation. The tubes that require a supplement are identified.

- Remove the caps from all bottles and tubes.
- Load the Reagents cassette into the instrument (Figure 13A).
- Lower the sipper carefully, and close the exterior fluidics door (Figure 13B). Change gloves before proceeding with depositing bead layer 3.

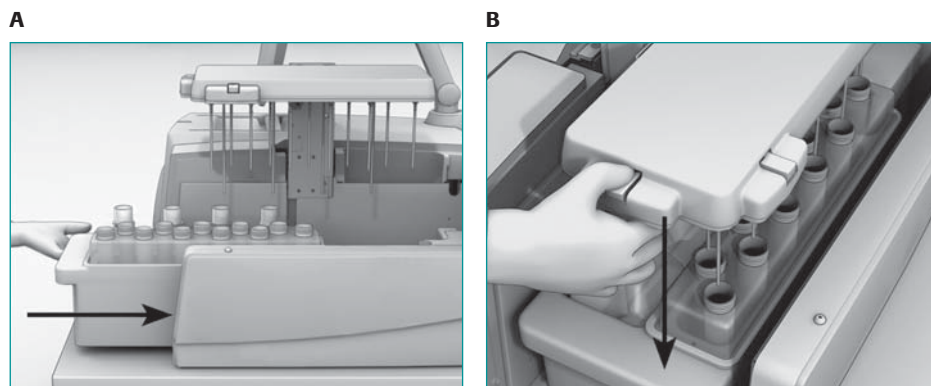


Figure 13: Loading the Reagents cassette into the instrument

- Step synchronization:** Return to the bead deposition procedure for the Enzyme Beads post-layer (bead layer 3; Section 3.2.5.3, above).

3.3.3 Clean the PicoTiterPlate Cartridge and the Camera Faceplate

- Step synchronization:** Perform the procedures described in this section and in Section 3.3.4 during the 5 minutes centrifugation of the Enzyme Beads post-layer (Section 3.2.5.3, step 9).

Sensitive camera face: Always be extremely careful when handling or working near the camera face. Never touch the camera face with anything other than Zeiss moistened cleaning tissue or Lens paper from Thorlabs.

- Click the *Unlock* button from the Instrument Tab (Figure 14).



Figure 14: The Unlock button used to unlock the camera door

- Open the camera door by pulling gently on the handle as shown in Figure 15.

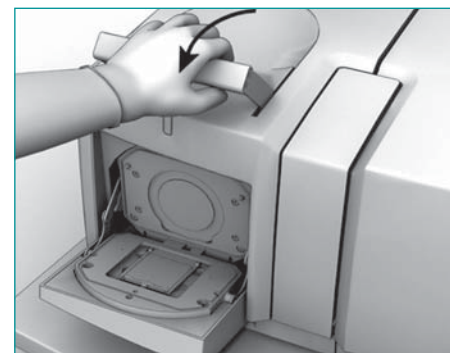


Figure 15: Opening the camera door

- Remove the spent PTP device from the PTP cartridge by first pressing the PTP frame spring latch (Figure 16A) to lift the frame from the cartridge, and then sliding out the used PTP device (Figure 16B).

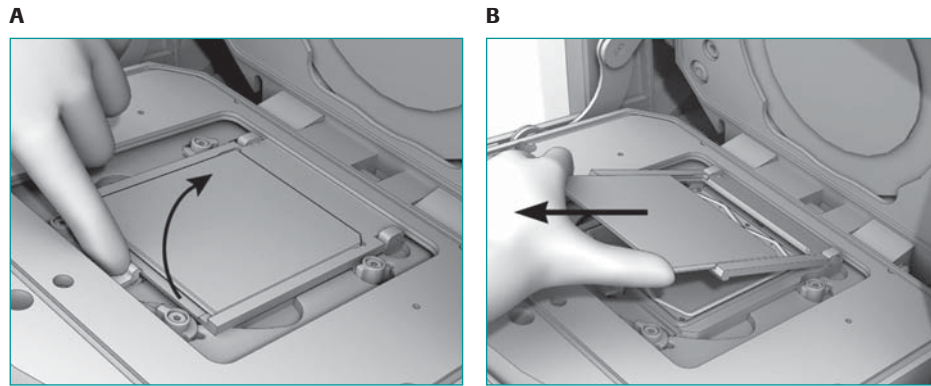


Figure 16: Removing the used PicoTiterPlate device

- Close the PTP frame and, using a pair of plastic forceps provided in the accessory set, remove the used PTP cartridge seal from the PTP cartridge (Figure 17A).
- Wet a Kimwipe with 50% ethanol and wipe the surface of the cartridge to remove any bead and reagent residue (Figure 17B). Allow the cartridge to air dry completely.

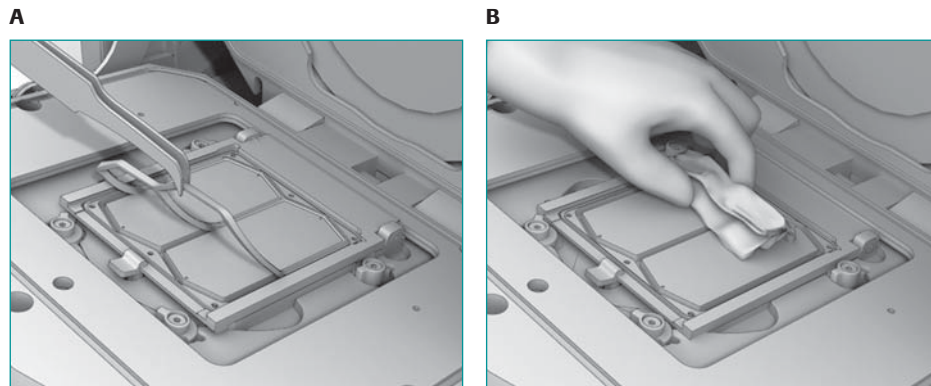


Figure 17: Removing the PTP cartridge seal and wiping the PTP cartridge

- Use a new Zeiss pre-moistened cleaning tissue to gently wipe the camera faceplate.
- Allow the camera faceplate to air dry completely.
- Moisten a Kimwipe with a 10% user-prepared solution of Tween-20, and wipe the surface of the PTP cartridge with it.

3.3.4 Load and Set the Run Script and Other Run Parameters (without LIMS)

This section describes how to manually set up and launch a sequencing Run. However, if the Genome Sequencer FLX Instrument is connected to a Laboratory Information Management System (LIMS), the Run Wizard will seek the information that describes the Run from your LIMS after you enter the PTP device ID.

- On the instrument computer, the GS Sequencer main window is displayed, with the Instrument Tab active (Figure 18).



Figure 18: Upper portion of the GS Sequencer software main window, with the Instrument tab active

- Click the Start button in the Global Action area.
 - This opens the Run Wizard's first window: Choose a procedure (Figure 19).

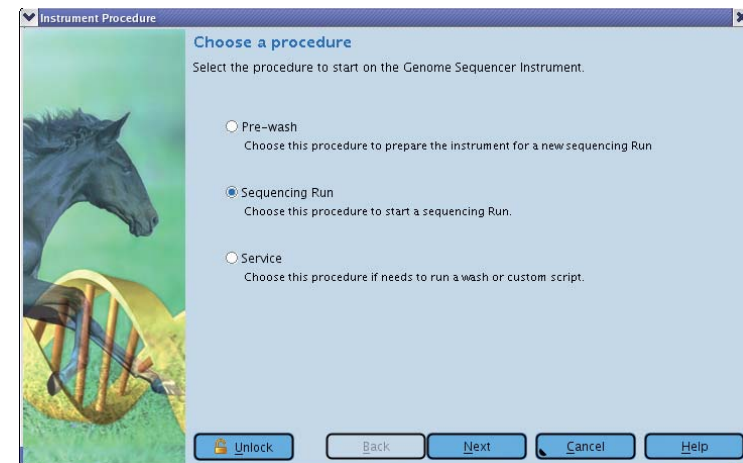


Figure 19: The Run Wizard's first window: Choose a procedure

Sequencing Method Manual

- In the Run Wizard's first window, select the Sequencing Run option, and click the Next button.

▶ This opens the Run Wizard's second window: Enter IDs and barcodes (Figure 20).

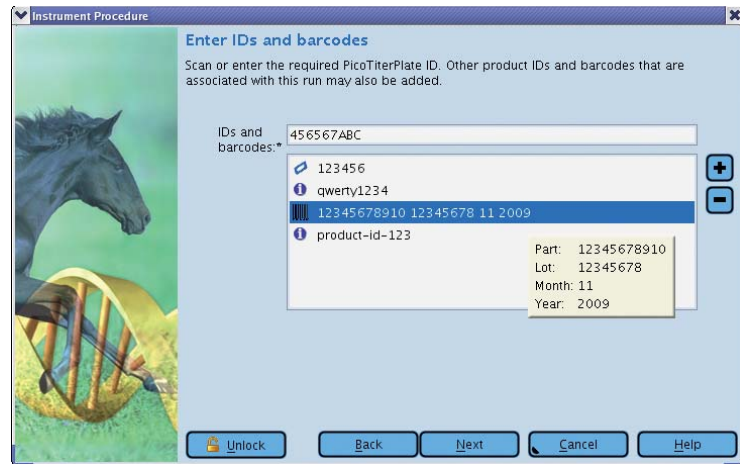


Figure 20: The Run Wizard's second window: Enter IDs and barcodes



- Enter the barcode of the PicoTiterPlate device to be used in this Run. You may also enter the product IDs or barcodes of other materials associated with this sequencing Run (e.g. Library Prep, emPCR, and Sequencing Kits); you will be asked for this information if you call Roche Customer Support for help if you encounter any difficulties with your Run. When you have entered all the information, click Next.

▶ This opens the Run Wizard's third window: Enter Run name and Run Group (Figure 21).

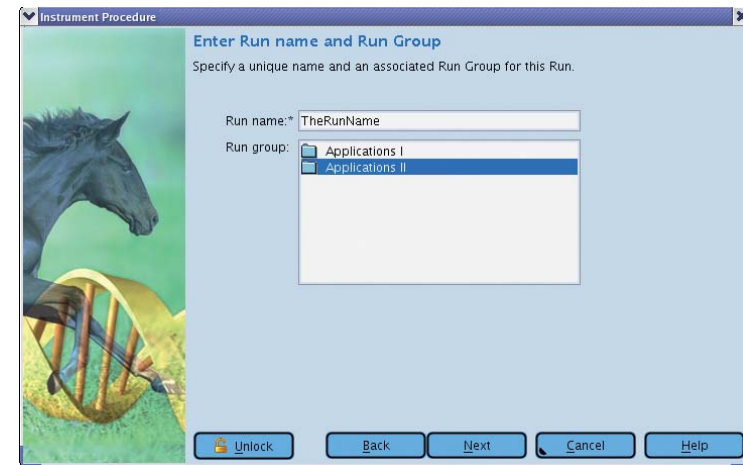


Figure 21: The Run Wizard's third window: Enter Run name and Run Group



- 5 Enter a specific, unique name for this Run. Then find and select your Run Group in the Run group list. Click Next.

► This opens the Run Wizard's fourth window: Choose sequencing kit (Figure 22).

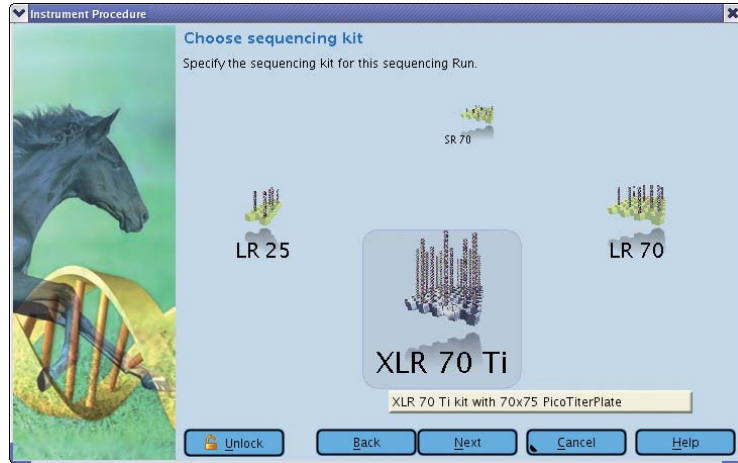


Figure 22: The Run Wizard's fourth window: Choose sequencing kit



- 6 Select the type of Sequencing kit to be used in this Run, and click Next.

► For the GS FLX Titanium chemistry, the only valid choice is XLR70Ti.

► This opens the Run Wizard's fifth window: Choose PicoTiterPlate type (Figure 24).

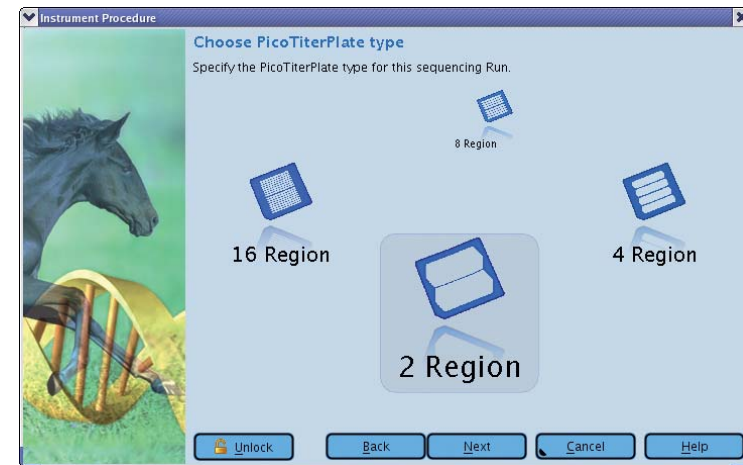


Figure 23: The Run Wizard's fifth window: Choose PicoTiterPlate type

- 7 Select the bead loading gasket to be used in this Run, and click Next.

► This opens the Run Wizard's sixth window: Choose number of cycles (Figure 24).

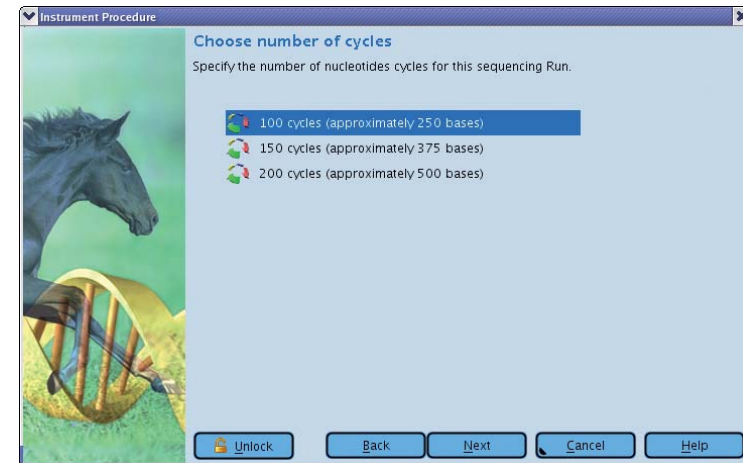


Figure 24: The Run Wizard's sixth window: Choose number of cycles



Sequencing Method Manual

- 8 Select the number of nucleotide cycles appropriate for this Run, and click Next.
 - ▶ Only Run lengths matching the Kit previously selected are available.
 - ▶ On the GS FLX Titanium chemistry,
 - ▶ 200 cycles will produce reads of about 350 – 450 bp
 - ▶ 150 cycles, about 275 – 325 bp
 - ▶ 100 cycles, about 200 – 250 bp
 - ▶ This opens the Run Wizard's seventh window: Choose Run Processing type (Figure 25).

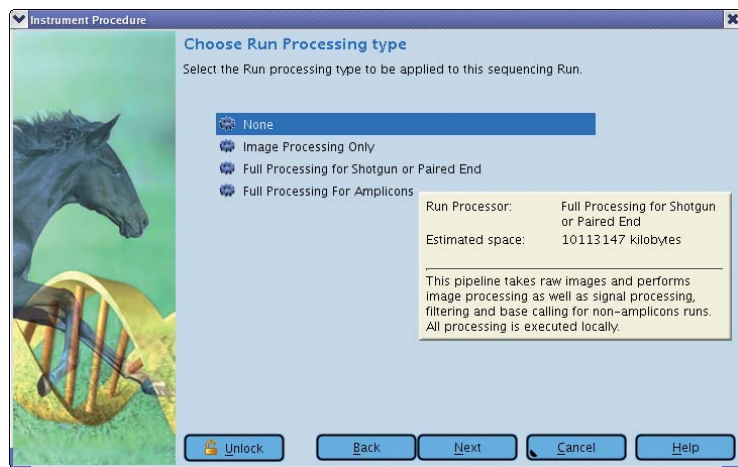


Figure 25: The Run Wizard's seventh window: Choose Run Processing type



- 9 Select the data processing scheme appropriate for this Run, and click Next.
 - ▶ Four data processing schemes are available on the Genome Sequencer FLX System (see the *Genome Sequencer System Software Manual* for a complete description of the Genome Sequencer FLX System's data processing and analysis processes, including a description of when to use each of these options):
 - ▶ **Full processing for Shotgun and Paired End:** The instrument will capture images during the Run, process them into "raw wells" data files (during Run-time), and then further process these into read flowgrams and basecalls with associated per-base quality scores. The resulting read flowgrams can be used later, in data analysis, to generate the consensus sequence for the DNA sample, either by *de novo* assembly of the reads, or by mapping them to a reference sequence. Select this processing type if sequencing a Rapid, Paired End (any span), cDNA, or a General library, or a Control DNA Bead run.
 - ▶ **Full processing for Amplicons:** This will do the same as the Full processing option except that it will use data processing algorithms that are specially tuned for Amplicon sequencing. Select this option if sequencing an Amplicon library and performing full Run-time data processing on it.
 - ▶ **Image processing only:** The instrument will only capture images during the Run, and process them into "raw wells" data files (during Run-time). The read flowgrams and basecalls with per-base quality scores must then be produced later (standard or "Amplicon" Signal Processing, as applicable).

After data processing has been completed, data analysis can be performed. This will consist either of determining the consensus sequence for the DNA sample or of amplicon variant analysis.
 - ▶ **No processing:** The instrument will only capture and store raw images for this Run. All steps of data processing must then be carried out separately.
 - ▶ This opens the Run Wizard's eighth window: Request data backup (Figure 26).



Figure 26: The Run Wizard's eighth window: Request data backup



- 10 Select the "Backup Run and Processor data upon completion" checkbox, and click Next.
 - ▶ With this checkbox selected, the data from the Run will be automatically backed up at the end of the Run (to a storage location pre-determined by an Administrator. See Appendix).
 - ▶ This opens the Run Wizard's ninth window: Run comments (Figure 27).

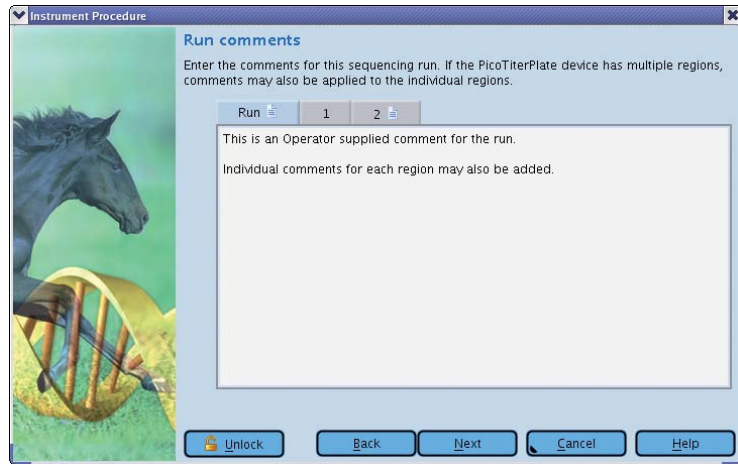



Figure 27: The Run Wizard's ninth window: Run comments



- 11 Enter any comments about the Run, and click Next.
 - ▶ This opens the tenth, and last, window: Insert new PicoTiterPlate device (Figure 28).



Figure 28: The Run Wizard's tenth, and last window: Insert new PicoTiterPlate device

-  **Step synchronization:** Return to the bead deposition procedure for the PPIase Beads layer (bead layer 4, Section 3.2.5.4).

3.3.5 Insert the PicoTiterPlate Device and Launch the Sequencing Run

- 1 Install the cartridge seal as described below:
 - a. Make sure that the square ridge on the seal is facing up (see Figure 29A), and drop the seal in the cartridge groove (Figure 29B).
 - b. If necessary, gently tap the seal into place with a gloved hand. DO NOT wipe the seal with anything, not even with a Kimwipe, as this could damage or stretch the seal.

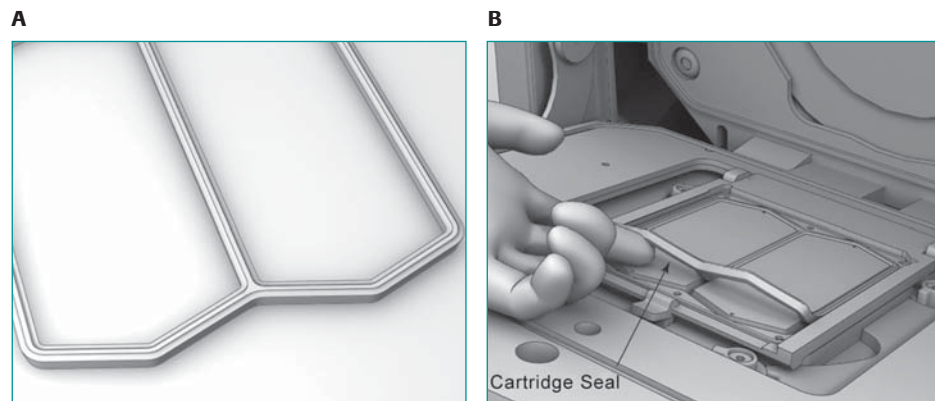


Figure 29: Orientation and placement of the cartridge seal

- 2 Press the PTP cartridge spring latch (Figure 30A) to lift the PTP frame from the cartridge.
- 3 When centrifugation of the bead layer 4 is complete, remove the BDD from the centrifuge, and discard the BDD port seals that cover the loading ports and air vents.
- 4 With a pipettor, gently draw out and discard all the supernatant from the centrifuged bead layer 4, through the port holes in the BDD top.
- 5 Remove the PTP device from the BDD, as follows:
 - a. Rotate down the latches of the BDD to unfasten them.
 - b. Carefully remove the BDD top.
 - c. Gently lift off and discard the bead loading gasket.
 - d. Remove the PTP device, being careful to handle it only by the edges.
- 6 Slide the PTP device into the PTP cartridge frame, face down. Make sure that the PTP device notch is on the lower right hand corner, matching the notch in the frame (Figure 30B).

- 7 Close the PTP frame, making sure it is properly secured by the latch (Figure 30C).
- 8 Wipe the back of the PTP device with a Kimwipe. Use a downward motion, toward the camera faceplate, to avoid sliding the PTP device within the frame, or out of it.
- 9 Close the camera door (Figure 30D).

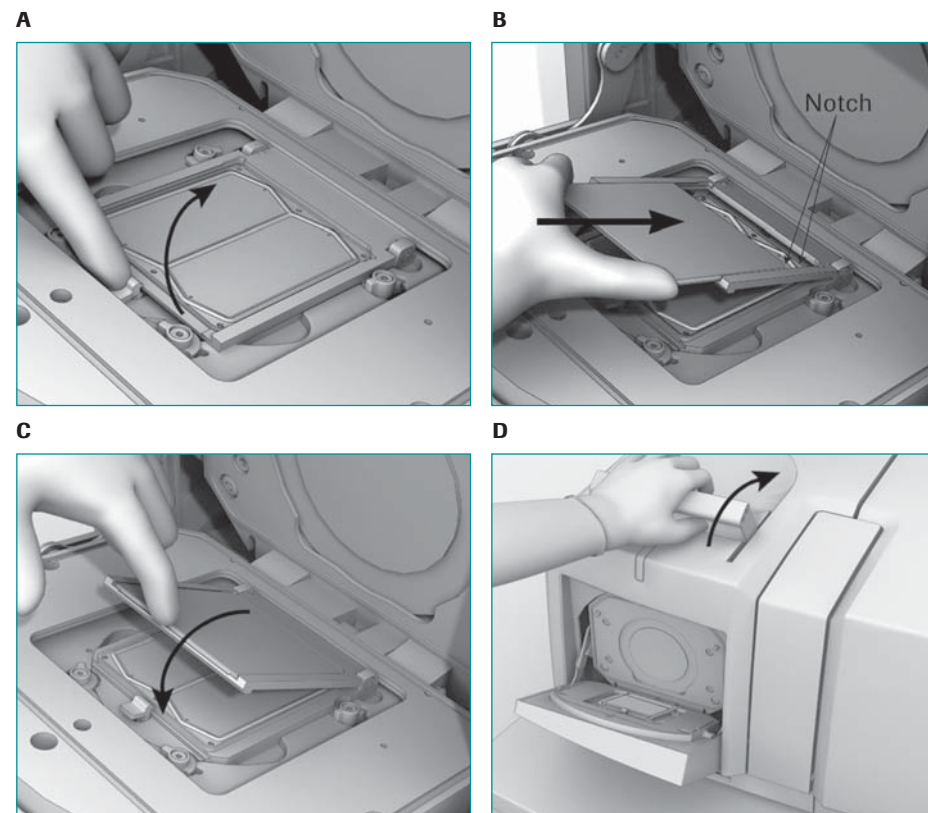


Figure 30: Loading a 70 × 75 mm PTP device into the PTP frame, in the cartridge

- 10 Click the Start button in the Run Wizard's Insert new PicoTiterPlate device window (Figure 27) to start the sequencing Run.
 - ▶ The Operator can monitor the progress of a sequencing Run by viewing the Instrument status and the data images as they are being captured by the camera: Thumbnail images will appear under the progress bar in the Instrument tab during the Run (Figure 31).
 - ▶ If the Start button is still grayed out, check for "x" in a red circle icons in the Run Wizard window, and address any problems that may be reported by the sensors.

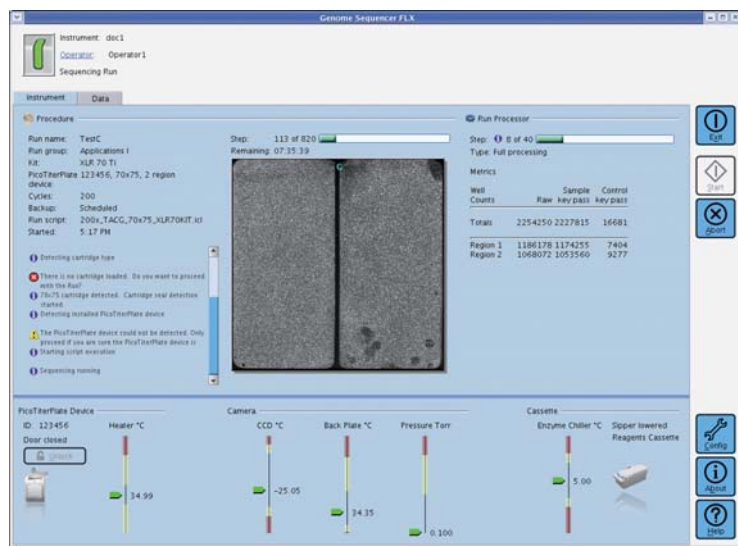


Figure 31: The Instrument tab of the GS Sequencer application main window during a sequencing Run

- 11
 - ▶ Monitor the instrument until the sequencing Run is under way and the Status LED located above the camera door on the instrument is blinking green (also shown at the upper-left corner of the GS Sequencer window). If the instrument encounters any problems during the initiation of the Run, a message describing the issue will appear in the Status area of the GS Sequencer window, and user intervention will be required.
 - In most cases, the software will offer to Abort or Proceed with the Run. If you are certain that the warning is unwarranted then you can decide to proceed.
 - In a few special cases, however, you will not be offered the possibility to proceed. If this happens, you must restart the software, as follows:
 - ▶ Close the GS Sequencer application.
 - ▶ Double-click the *systemStart* icon, located on the desktop.
 - ▶ Re-launch the GS Sequencer, and set up your Run again.
 - ▶ There is an *Abort* button available in the Global Action area of the GS Sequencer main window (Figure 31), which can be clicked if a problem occurs during the Run. The Abort Run dialog box will ask you to verify that you want to stop the Run; if you confirm, the Run will be immediately terminated. There is no procedure for pausing and resuming a Run. If a Run is aborted, follow the abort with a Pre-wash before proceeding with another sequencing Run.
- 11 When the sequencing Run is complete, a message will appear in the Status area of the GS Sequencer application window.

4. Appendix: Network Setup for Sequencing Data Backup

Data transfer is usually setup at the time of instrument installation. It is important to setup a network data backup mechanism to archive sequencing Run data, because the data sets are large, and the storage capacity on the instrument limited.

To enable backup on a per Run basis, the instrument control software will execute a shell script at the completion of the Run. This script (`/usr/local/rig/bin/backupScript.sh`) is intended to be customer-configurable to execute any on-instrument process necessary to initiate the backup mechanism.

- ▶ The script will have one argument passed to it: the fully qualified path of the Run directory.
- ▶ The only other requirement of the script is that its return value reflect the outcome of the backup procedure. This way, the instrument control GUI (the GS Sequencer application; the Data tab) can indicate to the user whether or not a particular Run's backup procedure was successful. Thus, the script should be modified such that the variable "RET_VAL" is set to 0 if the backup was successful and to 1 if it was not.

Note that if Run backup was selected during setup, and the backup either did not take place or failed, the user will not be allowed to delete the Run. This behavior is described in the section on the Data tab of the GS Sequencer application, in the *Genome Sequencer FLX System Software Manual*.

The Run data consists of image data, log files, and possibly image and signal processing data, if the "Image Processing Only" or one of the "Full Processing" data processing schemes was chosen during Run setup. Table 13 shows the approximate size of the data sets created during a sequencing Run for each sequencing kit type, Run script, and data processing option (assuming the largest bead loading regions available on the PTP device).

Sequencing Kit	Number of Cycles	Raw Images Only	Raw Images Plus Image Processing	Raw Images Plus Full Processign
XLR70	200	28 Gb	30 Gb	37 Gb
XLR70	150	21 Gb	23 Gb	28 Gb
XLR70	100	14 Gb	18 Gb	18 Gb

Table 13: Approximate amount of data generated by the XLR70 sequencing kit for each Run script and data processing option. These numbers assume that the 2 regions gasket is used

Table of Contents

1.	Workflow	2		
2.	Before You Begin	2		
2.1	What You Should Have Before Starting.....	2		
2.1.1	Sample	2		
2.1.2	Required Genome Sequencer FLX System Equipment and Reagents.....	2		
2.1.3	GS Sequencing Kit	2		
2.1.4	Choosing the Size and Number of PTP Regions for Your Sample	2		
2.1.5	Reagents and Titanium Bead Buffer	3		
3.	Procedure	3		
3.1	The Pre-Wash	3		
3.1.1	Launch the Pre-Wash.....	5		
3.2	PicoTiterPlate (PTP) Device Preparation.....	6		
3.2.1	Prepare Bead Buffer 2 (BB2).....	6		
3.2.2	Prepare the PicoTiterPlate and Bead Deposition Devices.....	6		
3.2.3	Prepare the Beads	7		
3.2.3.1	Prepare the Packing Beads.....	7		
3.2.3.2	Prepare the DNA Beads (Sample and Control).....	7		
3.2.3.3	Prepare the Enzyme and PPIase Beads (Bead Layers 1, 3 & 4)	8		
3.2.3.4	Combine the DNA and Packing Beads (Bead Layer 2).....	8		
3.2.4	Assemble the BDD with the PTP Device and Bead Loading Gasket	9		
3.2.5	Deposit the Four Layers of Beads on the PTP Device.....	9		
3.2.5.1	Deposit Bead Layer 1: the Enzyme Beads Pre-Layer	10		
3.2.5.2	Deposit Bead Layer 2: the DNA and Packing Beads	10		
3.2.5.3	Deposit Bead Layer 3: the Enzyme Beads Post-Layer	11		
3.2.5.4	Deposit Bead Layer 4: the PPIase Beads	11		
3.3	The Sequencing Run	12		
3.3.1	Remove the Pre-Wash Cassette and Clean the Fluidics Area Deck.....	12		
3.3.2	Prepare and Load the Sequencing Reagents Cassette.....	12		
3.3.3	Clean the PicoTiterPlate Cartridge and the Camera Faceplate	13		
3.3.4	Load and Set the Run Script and Other Run Parameters (without LIMS).....	14		
3.3.5	Insert the PicoTiterPlate Device and Launch the Sequencing Run	19		
4.	Appendix: Network Setup for Sequencing Data Backup	21		

**For Life Science Research Only.
Not for Use in Diagnostic Procedures.**

454, 454 LIFE SCIENCES, 454 SEQUENCING, GS FLX, GS FLX TITANIUM, GS JUNIOR, EMPCR, PTP, PICOTITERPLATE, REM, NIMBLEGEN, SEQCAP, FASTSTART, CASY, and INNOVATIS, are trademarks of Roche.

Other brands or product names are trademarks of their respective holders.

Published by

454 Life Sciences Corp.
A Roche Company
Branford, CT 06405

© 2010
454 Life Sciences Corp.
All rights reserved.

©1110