454 SEQUENCING

Roche

Sequencing Method Manual for the GS FLX+ Instrument

GS FLX+ Series — XL+ Kit

May 2011

Instrument / Kit

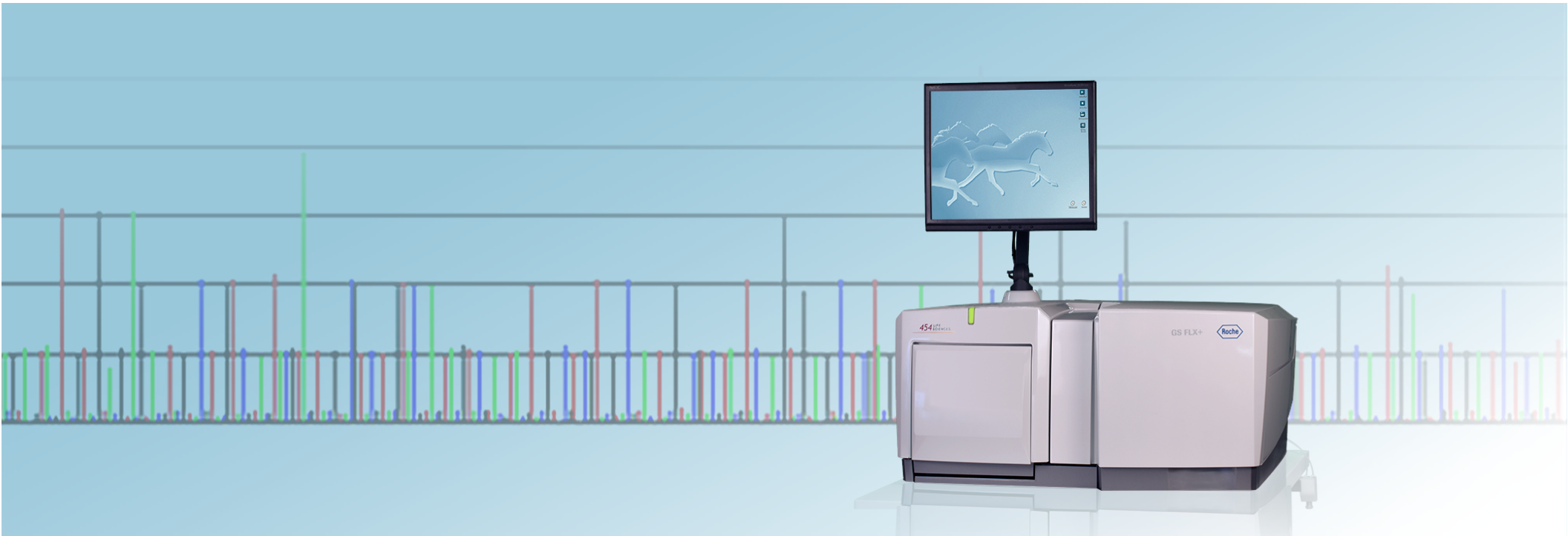
GS Junior / Junior

✓ **GS FLX+ / XL+**

GS FLX+ / XLR70

GS FLX / XLR70

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



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 an XL+ 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.

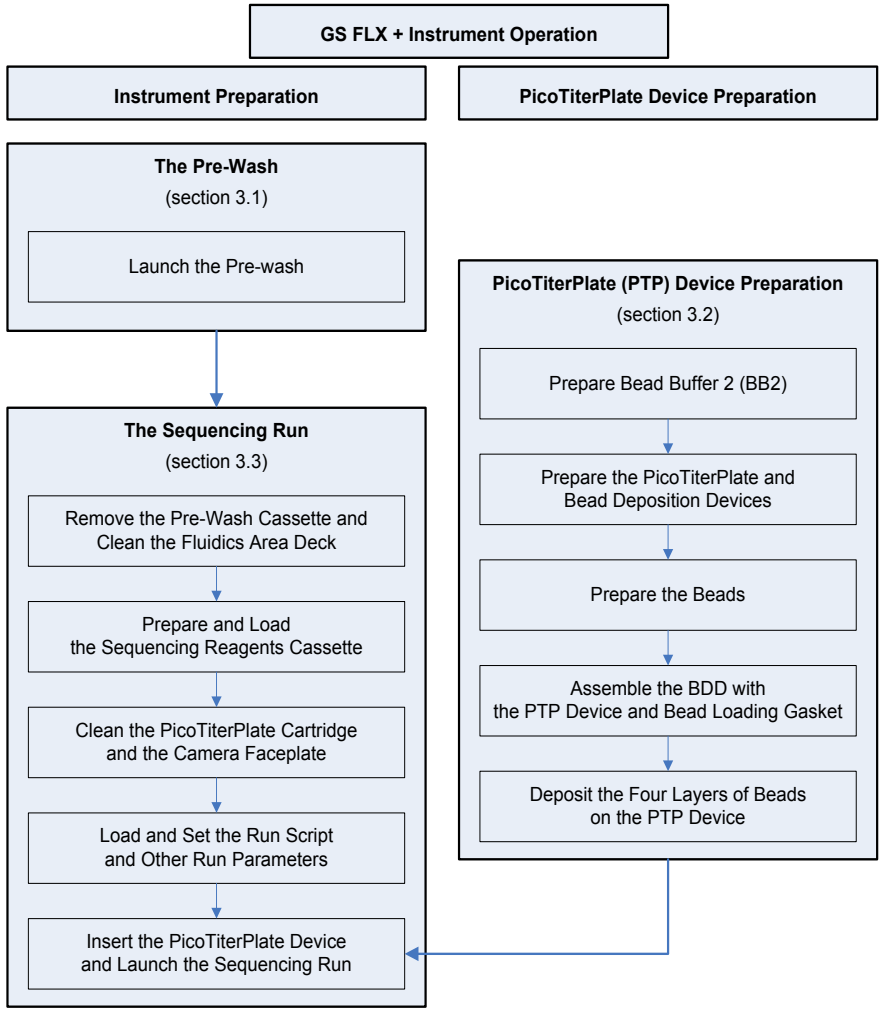


Figure 1: Workflow of a sequencing Run performed on the GS FLX+ Instrument, with the GS FLX Titanium Sequencing Kit XL+

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

Room temperature is +15 to +25°C.

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 Series — XL+ methods and the corresponding kits.

2.1.2 Required GS FLX+ System Equipment and Reagents

Refer to the customer access area of our web site, at www.454.com/my454 for the lists of materials and consumables required but not provided.

This method is for a GS FLX Titanium Sequencing Kit XL+ and a GS FLX+ Instrument.

2.1.3 Kits

The GS FLX Titanium Sequencing Kit XL+ is used in combination with the matching GS FLX Titanium PicoTiterPlate Kit 70 x 75. These kits provide reagents and components necessary for a single sequencing Run.

Refer to the customer access area of our web site, 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 Kit XL+ and PicoTiterPlate Kit support various bead loading region configurations. The size and number of PicoTiterPlate (PTP) regions to use depend on the sequencing throughput requirements of the experiment, 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 400 cycles sequencing Runs performed using good quality shotgun libraries. For example, column 4 shows the expected throughput of a full sequencing Run carried out with the longest run script available (400 cycles, generating reads up to 1 kbp in length), with all regions loaded.

Region Size	Regions per PTP Device	Bases per Region (Mbp)	Bases per Full PTP Device (Mbp)	Reads per Region (x 10^3)
Large	2	250-400	500-800	450-650
Medium	4	100-160	400-640	160-250
M/S	8	40-70	320-560	80-120
Small	16	16-25	240-400	25-40

Table 1: Gasket specification and throughput

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

- If the experiment aims to sequence 10 Mbp at 25-fold coverage, a total throughput of 250 Mbp is required. This can be obtained in a single large region of a PTP device.
- Sequencing 70 Mbp at a 30-fold coverage, requires just over 2 Gbp of total throughput and about 8 large regions, or 4 full sequencing Runs with the large region gasket.
- Resequencing 4 Mbp at 10-fold coverage yields a total 40 Mbp throughput and requires one single medium/small region.

2.1.5 Reagents and Titanium Bead Buffer

The Sequencing Reagents of the GS FLX Titanium Sequencing Kit XL+ 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 XL+ out of its frozen storage. Keep the Sequencing Enzymes sleeve at -15 to -25°C.
2. Remove the barrier bag from the Sequencing Reagents tray. Remove the tube of DTT from position 3 and place it with the Sequencing Enzymes sleeve at -15 to -25°C. Allow the other 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.

     or
   - Leave for 2.5 h at room temperature (+15 to +25°C).

     or
   - Leave at +2 to +8°C for up to 60 h (e.g. weekend).
3. Before beginning, place the bottle of Titanium Bead Buffer on ice.
4. For room temperature thawing (first two bullets above), 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 Enzymes sleeve to thaw on ice. Place the tube of DTT in a room temperature water bath to thaw, then leave at room temperature. Do not re-freeze the reagents.

3. PROCEDURE

3.1 The Pre-Wash

Under normal conditions of continuous operation, the instrument is kept running. 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 in 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 in 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.

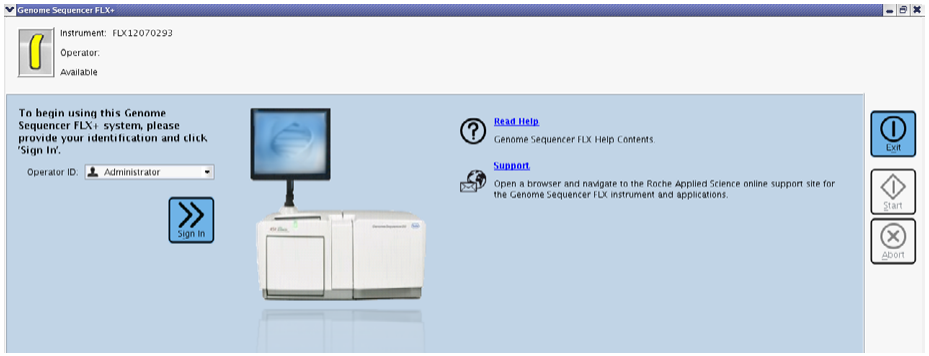


Figure 2: The login window of the GS Sequencer application

   - To login, select your user name in the Operator ID dropdown menu and click "Sign In". The main window of the GS Sequencer application will open (Figure 3).

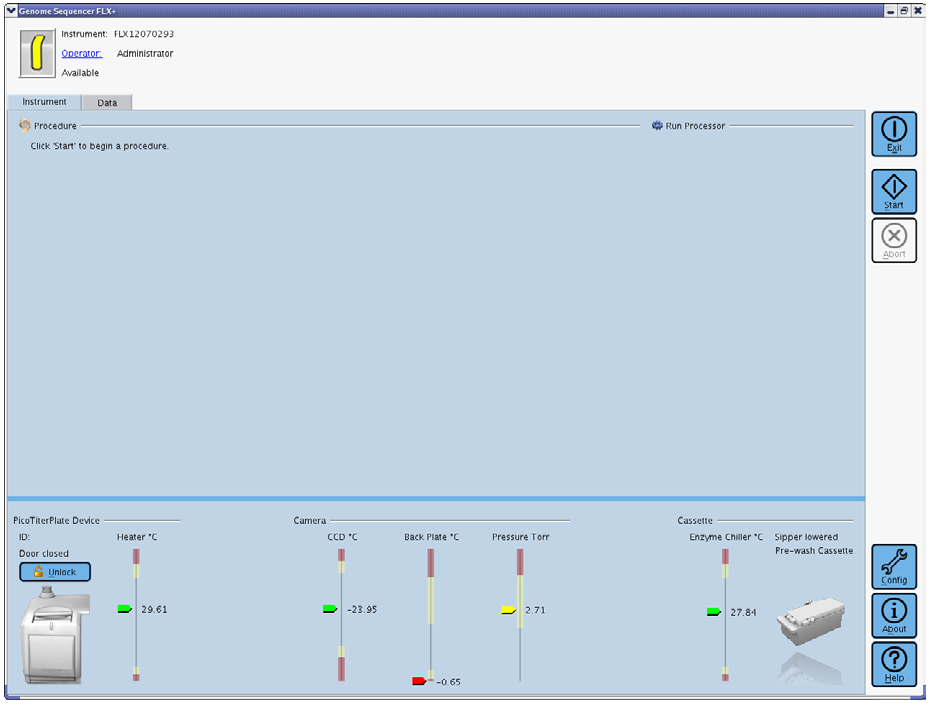


Figure 3: The GS Sequencer application window after an Operator logs in

3. Discard the spent reagents and clean the GS FLX+ Reagents Cassette, then perform the pre-wash:
   a. Open the exterior fluidics door and raise the sipper manifold completely (Figure 4**A**).
   b. Slide out the spent Reagents Cassette (Figure 4**B**).
   c. Discard the spent bottles and tubes.
   d. **Empty the GS FLX+ Waste Container and replace the cap.**
   e. Dry all the surfaces of the cassette with a paper towel.
   f. Replace the Sipper Tubes as described in the *GS FLX+ Instrument Owner's Manual*. Change gloves to avoid contaminating other components. Do not wipe the filter nor the Luer connector of the manifold as this risks introducing fibers into the system, which could clog fluidics valves.
   g. To prepare the pre-wash cassette, place the GS FLX+ Pre-wash Tube Holder on top of the Reagents Cassette and insert five long, grey Pre-wash Tubes on the left side and ten short grey Pre-wash Tubes on the right side in the pre-wash tube holder.
   h. Fill all the tubes to the top with Pre-wash Buffer (as in Figure 5**A**) and slide the cassette in the instrument, lower the manifold and close the fluidics door (Figure 5**B**).

Is it important to fill the Pre-wash Tubes to the top, as shown in Figure 5A, with Pre-wash Buffer for the Pre-wash to proceed correctly.

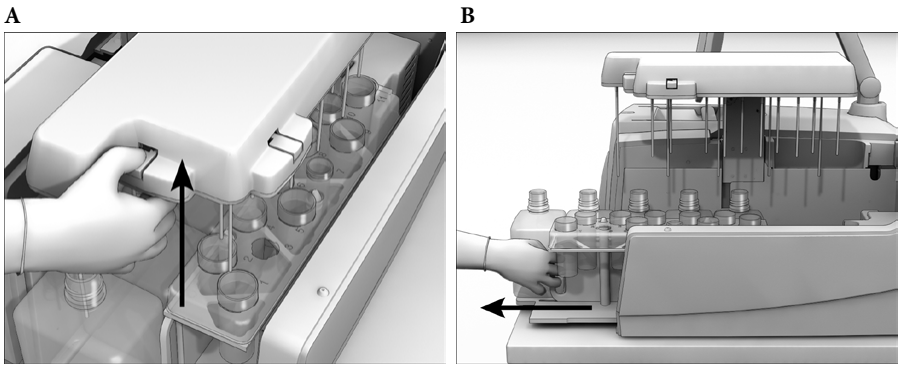


Figure 4: Removing the GS FLX+ Reagents Cassette (from the previous Run)

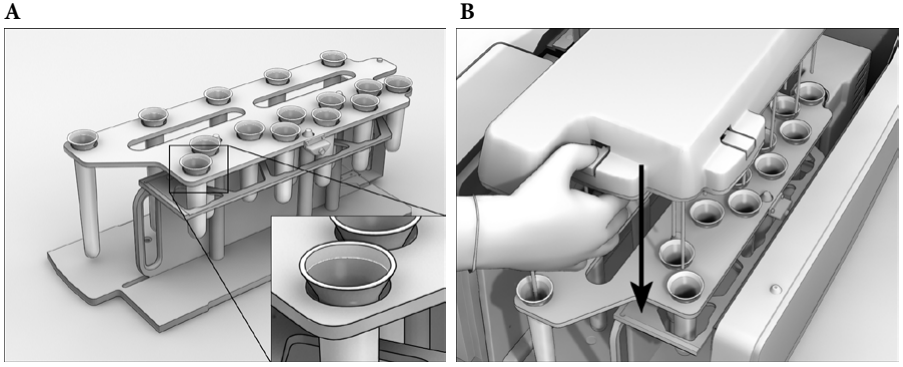


Figure 5: Filling the Pre-wash Tubes; loading before a pre-wash

4. 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 instruction is 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).

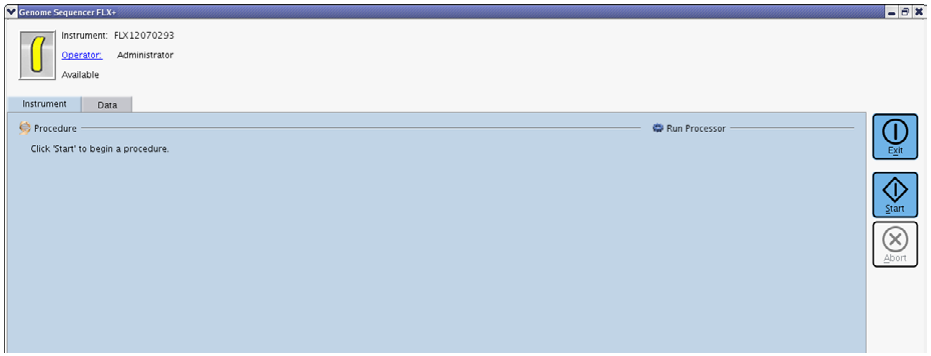


Figure 6: The Instrument tab of the GS Sequencer software

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

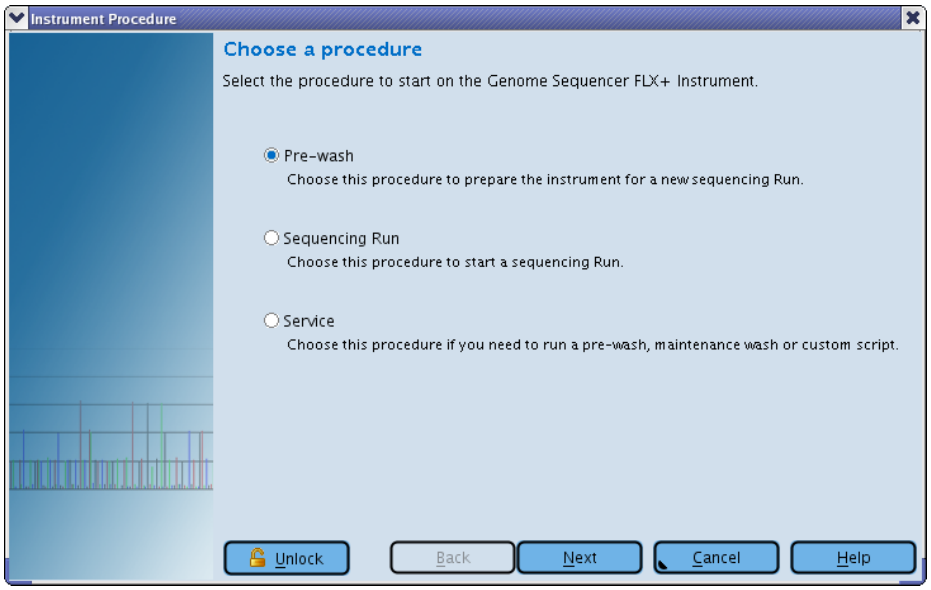


Figure 7: The Run Wizard's first window (Choose a procedure), with the Pre-wash option selected

3. 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).

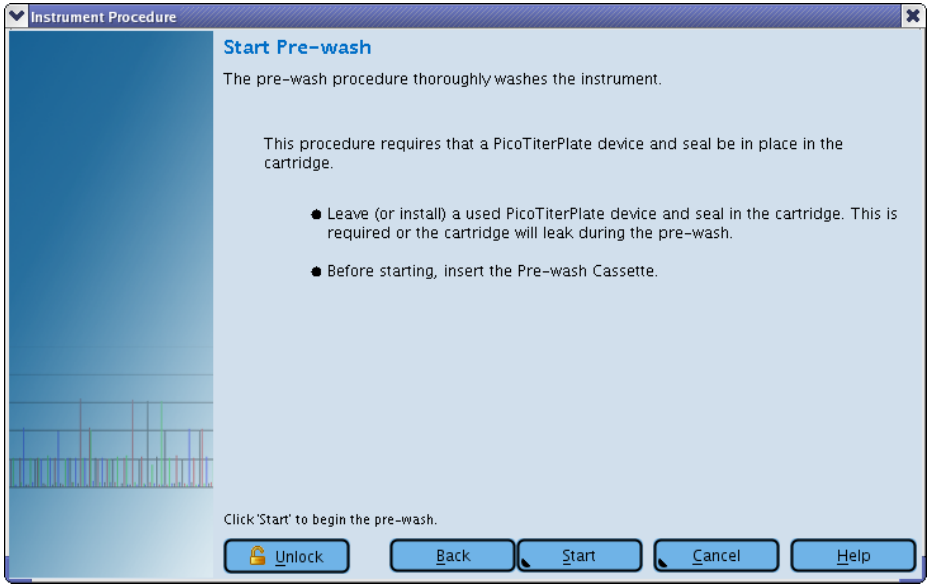


Figure 8: The Run Wizard's second window (last Wizard window for Pre-wash): Start Pre-wash

4. 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 Enzyme Bead Wash (EB Wash) and Bead Buffer 2 (BB2)

1. 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.
2. Pour **20 ml** of the buffer made in Step 1 in a 50 ml conical tube. Remove the tube of Apyrase from the ice. Leave the other components on ice until needed.
3. Add **6.8 µl** of Apyrase to the 20 ml supplemented Titanium Bead Buffer in the conical tube. Re-cap and label the conical tube "EB Wash". Mix gently by inversion, and place the conical tube on ice.
4. Add **30.6 µl** of Apyrase solution to the bottle of supplemented Titanium Bead Buffer. Re-cap and label the bottle "BB2". Mix gently by inversion, and place the bottle on ice.
5. Return the remainder of the Titanium Supplement CB and of the Apyrase on ice.

3.2.2 Prepare the PicoTiterPlate and Bead Deposition Devices

1. 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.
2. Write down the PTP device ID to enter the 6-digit number in the second window of the Run Wizard (Section 3.3.4).
3. Completely submerge the PTP device in BB2 at room temperature until you are ready to assemble and spin the Bead Deposition Device (BDD) (Section 3.2.5.1). Keep the rest of the BB2 on ice.
4. 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.
5. 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 GS FLX+ System contains four 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 have been prepared using the appropriate 454 Sequencing System procedures and kits. Before use, the sample DNA Beads will be spiked (Section 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 Package 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 four types of beads used in the GS FLX+ System

3.2.3.1 Prepare the Packing Beads

1. Wash the Packing Beads **three times** in **1 ml** of BB2 by centrifuging at **10,000 rpm** (9300 x *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 µl** of BB2 per tube and resuspend 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 2).
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/µl.
3. Vortex the DNA library beads and transfer the appropriate amount of beads into new 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 3).
5. Calculate the volume of supernatant to remove that will leave the volume indicated in column 4, 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.
6. Spin the beads for **1 minute** to form a pellet (use a minifuge or a microcentrifuge). 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 4, skip this centrifugation, as you have no volume to remove.
7. Set a pipettor to the volume calculated in Step 5, above. Being careful not to disturb the bead pellet, carefully and slowly remove the appropriate amount of supernatant and discard it.

Loading Region Size	Number of DNA Library Beads to Load per Region	Volume of Control DNA Beads (µl)	Target Final Volume, after Centrifugation (µl)
Large	2,000,000 (x 2)	20 (x 2)	50 (x 2)
Medium	790,000 (x 4)	10 (x 4)	30 (x 4)
Medium/Small	340,000 (x 8)	6 (x 8)	15 (x 8)
Small	125,000 (x 16)	3 (x 16)	10 (x 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 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	BB2 (µl)	Polymerase Cofactor (µl)	DNA Polymerase (µl)	Total Volume (µl)
All sizes	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 second 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	DNA Beads (µl)	DBIM (µl)	Total Volume (µl)
Large	50 (x 2)	950 (x 2)	1000 (x 2)
Medium	30 (x 4)	320 (x 4)	350 (x 4)
Medium/Small	15 (x 8)	140 (x 8)	155 (x 8)
Small	10 (x 16)	50 (x 16)	60 (x 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. However, make sure to change pipette tips to avoid contaminating them with one another.

1. Vortex the Enzyme Beads and the PPiase Beads and place them in a Magnetic Particle Concentrator (MPC) for at least **2 minutes** for the beads to pellet. Invert the MPC several times and wait at least **2 minutes** again. Carefully remove the supernatants and then remove the tubes from the MPC.
2. Wash the Enzyme Beads **three times** with **1 ml** of EB Wash for each tube of Enzyme Beads. Vortex and wash the beads using the MPC, as above.
3. After the third wash, add **1 ml** of EB Wash to each tube of Enzyme Beads. Vortex to resuspend the pellets. Keep on ice.
4. Wash the PPiase Beads **three times** with **1 ml** of BB2. Vortex and wash the beads using the MPC, as above.
5. After the third wash, add **500 µl** of BB2 to the tube of PPiase Beads. Vortex to resuspend the pellets. Keep on ice.
6. Prepare the beads for layers 1, 3, and 4 in three 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 EB Wash or BB2, as listed in Table 6. Vortex the washed Enzyme and PPiase Beads to get a uniform suspension before transferring.

Bead Layer	EB Wash or BB2 (µl)	Enzyme Beads (µl)	PPiase Beads (µl)	Total Volume (µl)
1	3230 (EB Wash)	570	---	3800
3	2460 (EB Wash)	1340	---	3800
4	3340 (BB2)	---	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	DNA Beads (µl)	Packing Beads (µl)	BB 2 (µl)	Total Volume (µl)
Large	1000 (x 2)	265 (x 2)	435 (x 2)	1700 (x 2)
Medium	350 (x 4)	100 (x 4)	210 (x 4)	660 (x 4)
M/S	155 (x 8)	45 (x 8)	125 (x 8)	325 (x 8)
Small	60 (x 16)	15 (x 16)	35 (x 16)	110 (x 16)

Table 7: Preparation of Bead Layer 2

2. Vortex the DNA and Packing Beads mixes and place them on the lab rotator for **at least 5 min** at room temperature. The unused packing beads can be discarded.

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. However, be careful NOT to allow the side with the wells to dry.
3. Place the PTP device onto the BDD base (Figure 9**A**), 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 9**B**). 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 9**C**) 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 9**D**). 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.

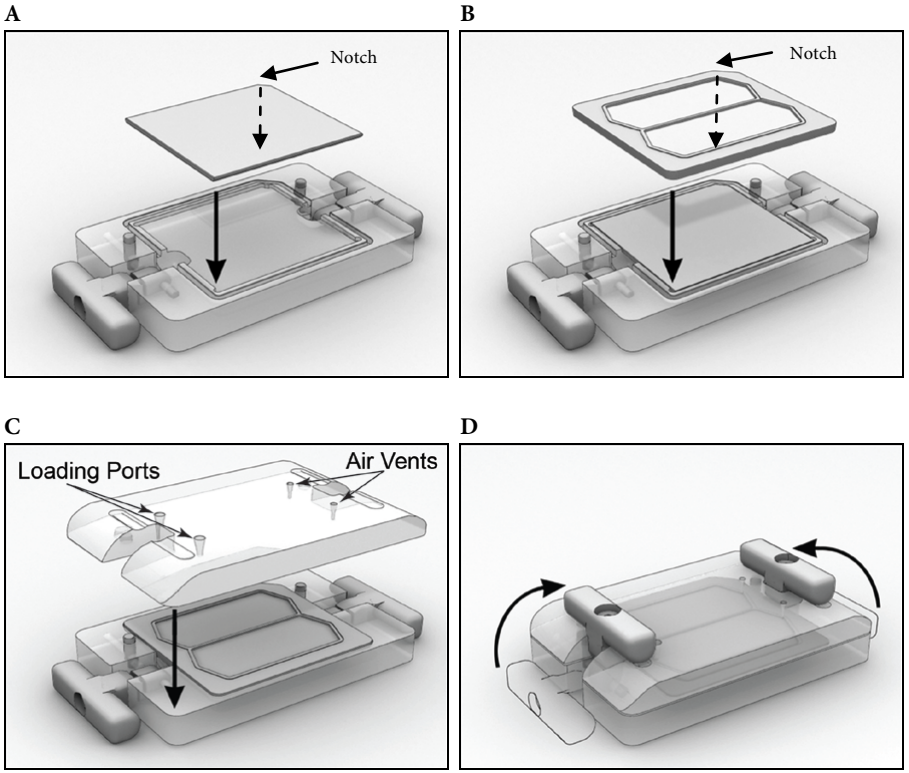


Figure 9: Assembly of the Bead Deposition Device

3.2.5 Deposit the Four Layers of Buffer/Beads on the PTP Device

Deposit the beads onto the PTP device by injecting the suspensions through the port holes of the assembled Bead Deposition Device (BDD), and then using centrifugal sedimentation to settle the suspensions to the bottom of the PicoTiterPlate wells. The PTP device is wetted first with BB2, and this process is then repeated for each of four layers:

- Wet the PTP device with Bead Buffer 2
- 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 the beads: 2 ml tip for Large regions, 1 ml tip for Medium and M/S regions, and 200 µl 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 Wet the PTP device: Bead Buffer 2

1. Using a pipette and a tip of the proper size, draw the appropriate amount of BB2 for the loading region size you are using per Table 8, and add the buffer through the loading ports.

Loading Region Size	Volume to Load (µl)
Large	1860 (x 2)
Medium	660 (x 4)
Medium/Small	325 (x 8)
Small	110 (x 16)

Table 8: Volume of BB2 to load per region for each bead loading gasket configuration

2. 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.
3. Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 x *g*** (2640 RPM for the Beckman Coulter X-12 or X-15 centrifuges).

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

1. Pipet out BB2 from the loading ports.
2. Vortex the bead suspension for Bead Layer 1 for 5 seconds to obtain a homogeneous suspension.
3. 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 9.

Loading Region Size	Volume to Load (µl)
Large	1860 (x 2)
Medium	660 (x 4)
Medium/Small	325 (x 8)
Small	110 (x 16)

Table 9: Volume of Bead Layer 1 to load per region for each bead loading gasket configuration

4. 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.

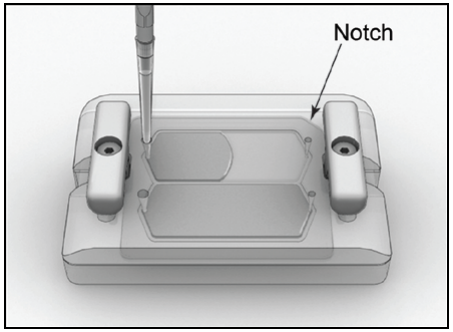


Figure 10: Filling the loading regions of the assembled Bead Deposition Device

5. Repeat Steps 1 to 3 for all loading regions of the PTP device.
6. Place both the assembled BDD and the BDD counterweight in centrifuge swinging baskets as appropriate for your centrifuge rotor, and place them on the rotor opposite each other.
7. Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 x *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.

3.2.5.3 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 Bead Layer 2 from the rotator.

4. Briefly spin the bead suspension for Bead 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 10.

Loading Region Size	Volume to Load (µl)
Large	1700 (x 2)
Medium	660 (x 4)
Medium/Small	325 (x 8)
Small	110 (x 16)

Table 10: Volume of Bead Layer 2 to load per region for each bead loading gasket configuration

6. 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. Centrifuge the loaded PTP device in the BDD for **10 minutes at 1620 x *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.

3.2.5.4 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 supernatant, for troubleshooting purposes in case the sequencing Run does not produce the expected number of reads.
4. Vortex the bead suspension for Bead 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 11.

Loading Region Size	Volume to Load (µl)
Large	1860 (x 2)
Medium	660 (x 4)
Medium/Small	325 (x 8)
Small	110 (x 16)

Table 11: Volume of Bead Layer 3 to load per region for each bead loading gasket configuration

For the third and fourth Bead 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. Promptly load the bead suspension 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. Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 x *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.

3.2.5.5 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 Bead 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 12.

Loading Region Size	Volume to Load (µl)
Large	1860 (x 2)
Medium	660 (x 4)
Medium/Small	325 (x 8)
Small	110 (x 16)

Table 12: Volume of Bead Layer 4 to load per region for each bead loading gasket configuration

5. 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. Centrifuge the loaded PTP device in the BDD for **5 minutes at 1620 x *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 4 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 11**A**).
3. Slide out the Pre-wash Reagents Cassette (Figure 11**B**).

A **B**

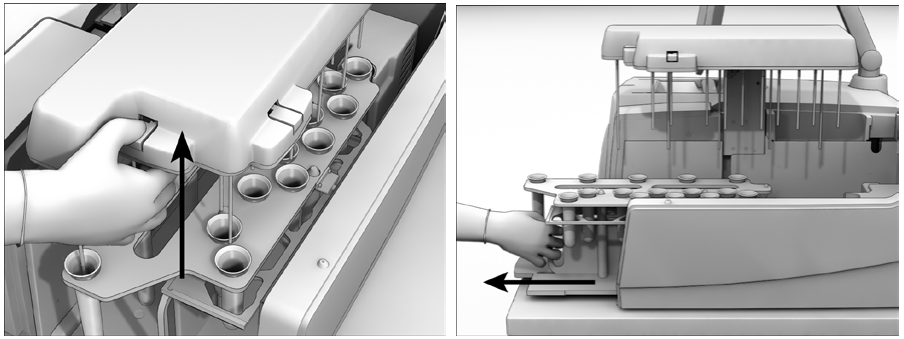


Figure 11: Removing the Pre-wash cassette from the instrument

4. Remove the grey 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 run.

5. Wipe all the 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 *GS FLX+ Instrument Owner's Manual*.

Step synchronization: Return to the bead deposition procedure for the DNA and Packing Beads layer (Bead Layer 2, Section 0).

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). Make sure that the DTT is completely resuspended before supplementing.

1. Retrieve the five bottles of Titanium Buffer CB stored at room temperature.
2. Add **13.2 ml** of Titanium Supplement CB and **2 ml** of DTT to **each** bottle of Titanium Buffer CB. Re-cap and gently invert the bottles to mix.
3. Retrieve the Sequencing Reagents tray from +2 to +8°C.
4. Dilute **5 µl** of the PPiase reagent in **45 µl** Inhibitor TW reagent in a 1.7 ml tube, and vortex.
5. Supplement the two reagents from the Sequencing Reagents tray with their appropriate additives, as listed below; then, change gloves.
   a. Add 32 µl of diluted PPiase (from Step 4) to the tube of "Inhibitor TW" (tray position 9, pink sticker).
   b. Add 675 µl of Apyrase to the tube of "Apyrase Reagent Buffer" (tray position 11, yellow sticker).
6. Make sure that all the caps are secure and 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).
7. Slide the empty Reagents Cassette at least half-way in the instrument, with the Sequencing Reagents tray platform placed on the right (Figure 12).

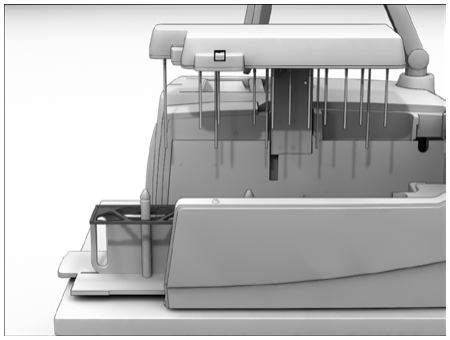


Figure 12: The empty Reagents Cassette in the GS FLX+ Instrument

8. Loosen the caps of all the bottles and tubes.
9. Place the five bottles of supplemented Titanium Buffer CB XL+ on the left side of the cassette (Figure 13**A**).
10. Place the Sequencing Reagents tray on its platform on the right side of the cassette, with the tube in position 11 resting on top of the retainer block (Figure 13**B**).

A **B**

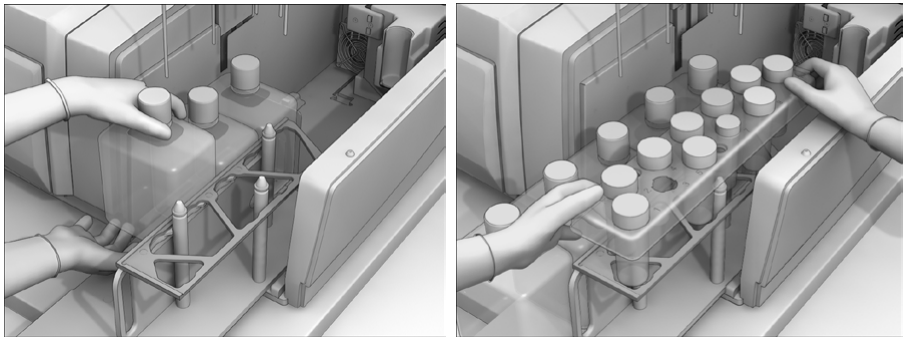


Figure 13: The Sequencing Reagents tray in the proper orientation

11. Remove the caps from the bottles and tubes, beginning by the ones in the back, avoiding handling caps over opened containers and touching the Sipper Tubes.
12. Slide the full Reagents Cassette completely in the instrument (Figure 14**A**), making sure that the tube of Apyrase (position 11) is properly engaged in the chiller. Lower the sipper carefully, and close the exterior fluidics door (Figure 14**B**). Change gloves before proceeding with depositing Bead Layer 3.

A **B**

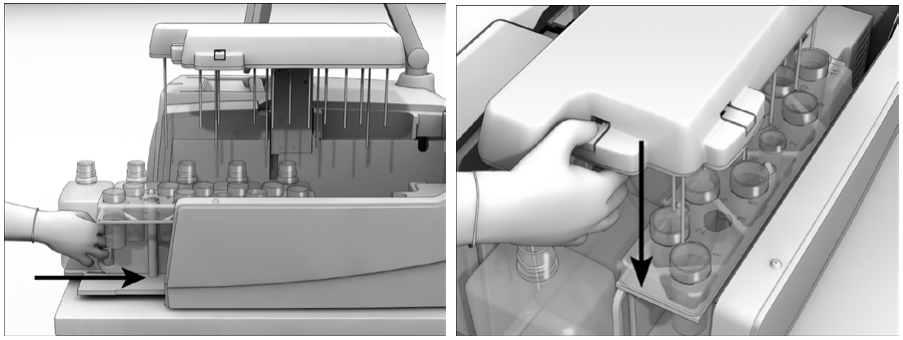


Figure 14: Loading the Reagents Cassette into the instrument

Step synchronization: Return to the bead deposition procedure for the Enzyme Beads post-layer (Bead Layer 3; Section 3.2.5.4).

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 3.2.5.4, Step 8).

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.

1. Click the *Unlock* button near the lower-left corner of the Instrument Tab (Figure 15).

Figure 15: The Unlock button used to unlock the camera door

2. Open the camera door by pulling gently on the handle as shown in Figure 16.

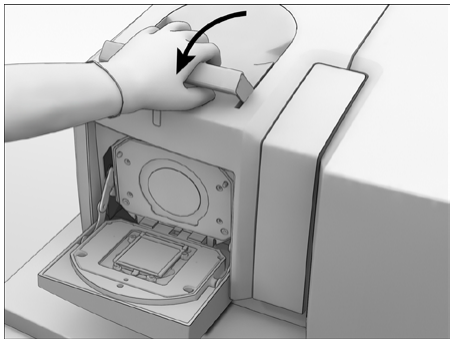


Figure 16: Opening the camera door

3. Remove the spent PTP device from the PTP cartridge by first pressing the PTP frame spring latch (Figure 17**A**) to lift the frame from the cartridge, and then sliding out the used PTP device (Figure 17**B**).

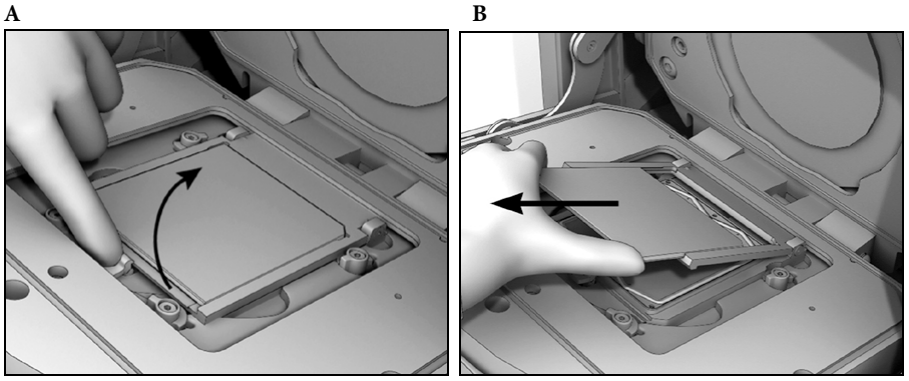


Figure 17: Removing the used PicoTiterPlate device

4. 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 18**A**).
5. Wet a Kimwipe with 50% ethanol and wipe the surface of the cartridge to remove any bead and reagent residue (Figure 18**B**). Allow the cartridge to air dry completely.

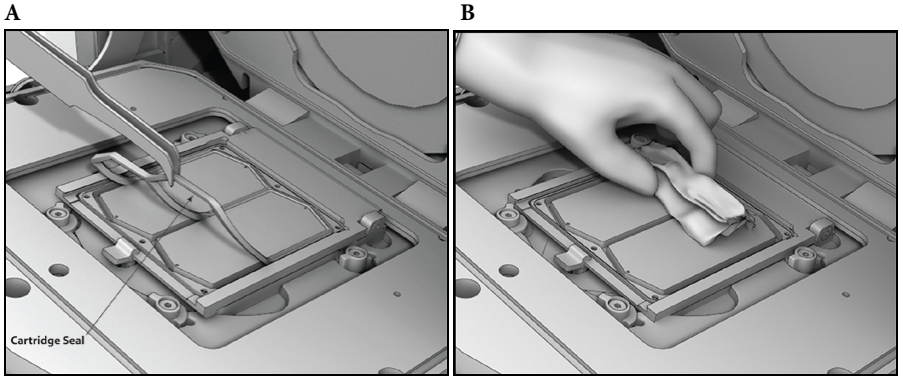


Figure 18: Removing the PTP cartridge seal and wiping the PTP cartridge

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 GS 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.
- Ensure there is enough free data space on the instrument for the run. Go to the Data tab and click on the instrument icon at the top of the list of Runs to view the information about the free space available. The total size of the raw images for a 2 regions, 400 cycle GS FLX Titanium Sequencing Kit XL+ Run is about 35 GB.

1. On the instrument computer, the GS Sequencer main window is displayed, with the Instrument Tab active (Figure 19).

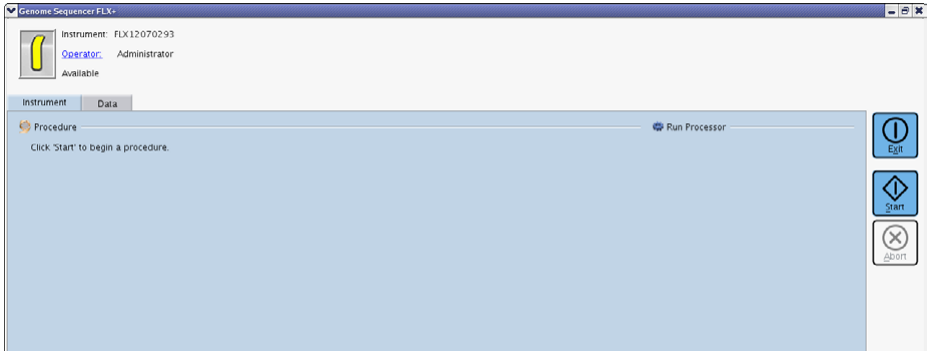


Figure 19: Upper portion of the GS Sequencer software main window, with the Instrument tab active

2. Click the Start button in the Global Action area.
   - This opens the Run Wizard's first window: Choose a procedure (Figure 20).

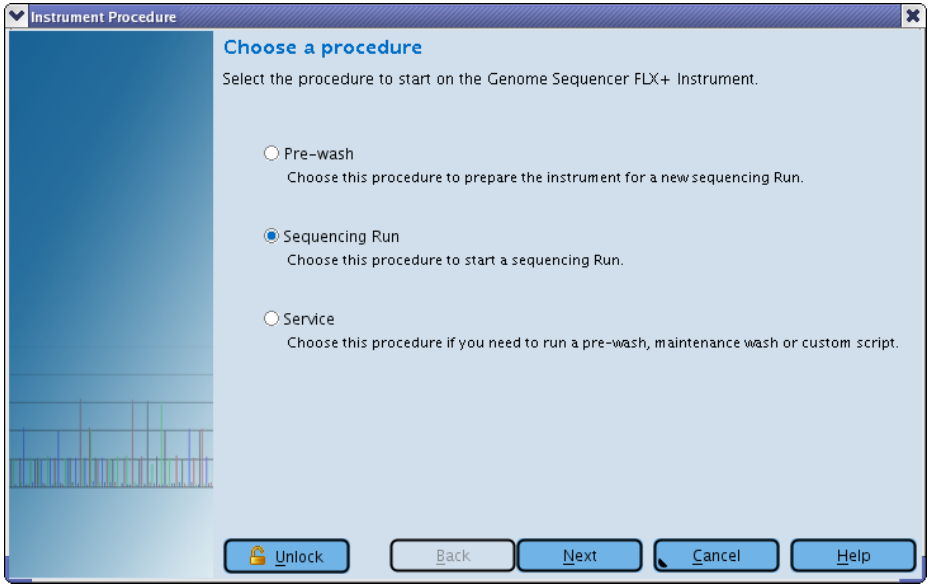


Figure 20: The Run Wizard's first window: Choose a procedure, with Sequencing Run option selected

3. 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 bar codes
   - Enter IDs and bar codes (Figure 21).

Figure 21: The Run Wizard's second window: Enter IDs and bar codes

4. Enter the bar code of the PicoTiterPlate device to be used in this Run. You may also enter the product IDs or bar codes 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 22).

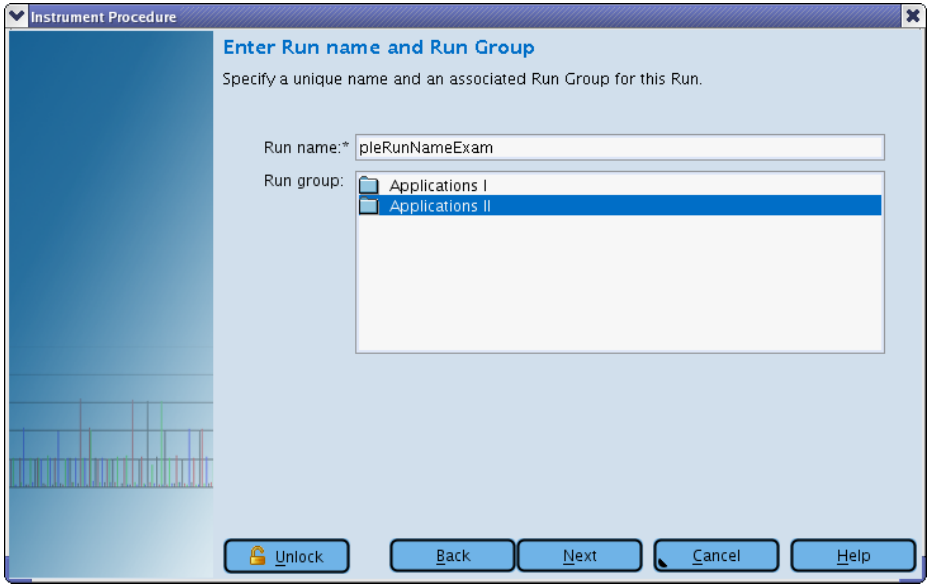


Figure 22: The Run Wizard's third window: Enter Run name and Run Group

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 23).

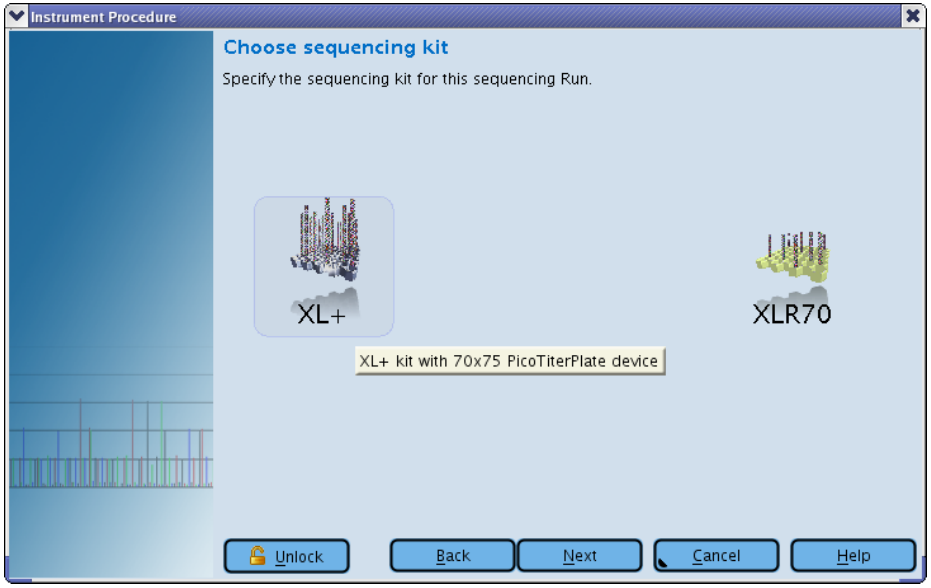


Figure 23: The Run Wizard's fourth window: Choose sequencing kit. Your selection will be highlighted.

6. Select XL+ and click Next.
   - This opens the Run Wizard's fifth window: Choose PicoTiterPlate type (Figure 24).

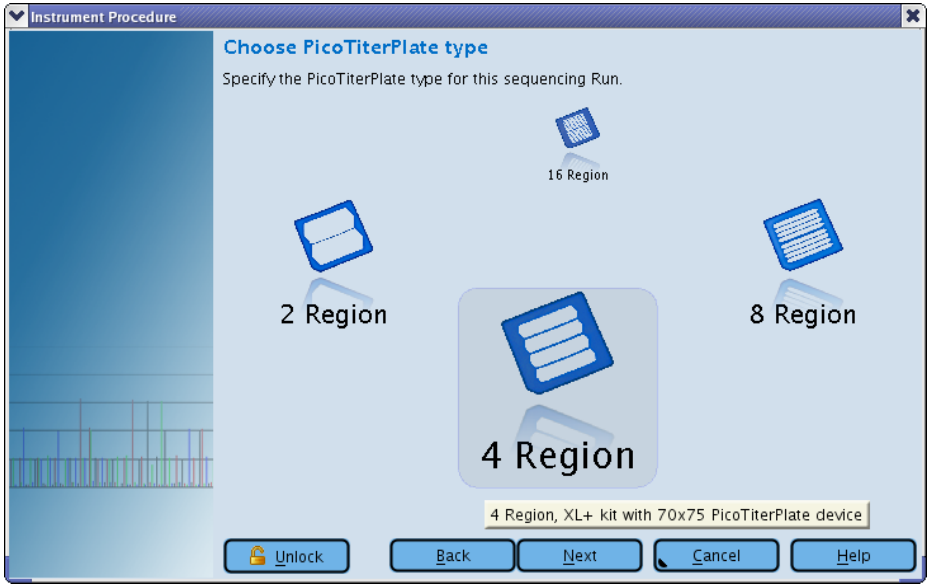


Figure 24: The Run Wizard's fifth window: Choose PicoTiterPlate type

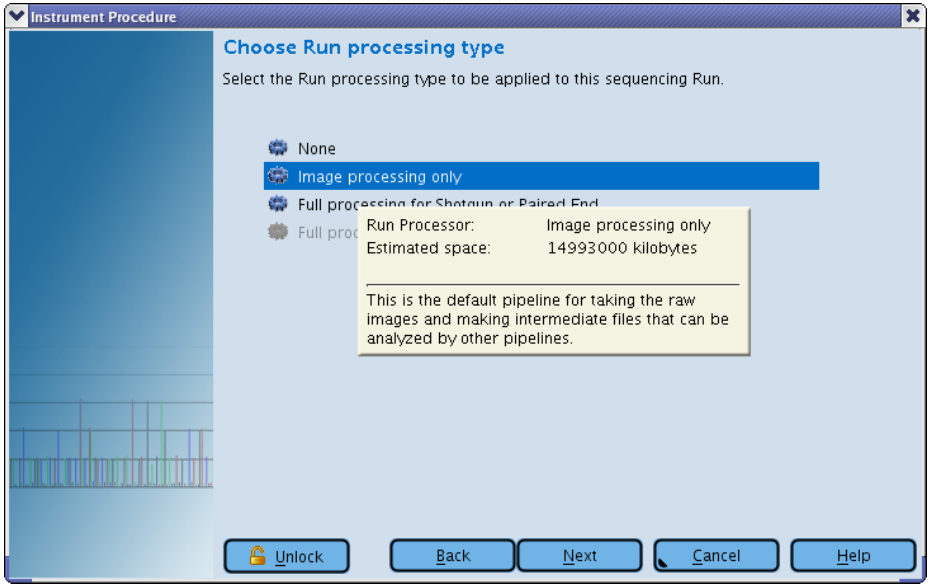


Figure 25: The Run Wizard's sixth window: Choose Run Processing type

7. Select the bead loading gasket to be used in this Run, and click Next.
   - This opens the Run Wizard's sixth window: Choose Run Processing type (Figure 25).

8. Select the processing scheme appropriate for this Run, and click Next.
   - Three data processing schemes are available on the GS FLX+ Instrument when using a GS FLX Titanium Sequencing Kit XL+ (see the *454 Sequencing System Software Manual* for a complete description of the GS 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. You may select this processing type if sequencing a Rapid or a cDNA Rapid library; be aware, however, that the computations under this option will take a very significant amount of time on the computer resources available on the GS FLX+ Instrument (up to a week or more).
     - **Image processing only**: The instrument will only capture images during the Run, and process them into "raw wells" data files; this will start during Run-time and may complete up to 3 hours or more after completion of the fluidics part of the sequencing Run. The read flowgrams and basecalls with per-base quality scores must then be produced later.

- **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 seventh window: Request data backup (Figure 26).

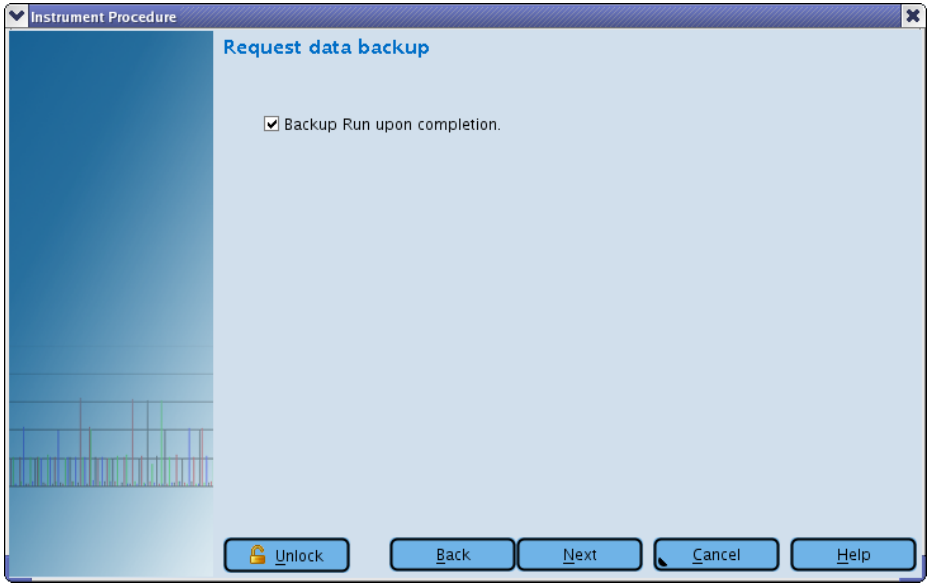


Figure 26: The Run Wizard's seventh window: Request data backup

9. Select the "Backup Run upon completion" checkbox, and click Next.

   - With this checkbox selected, the data from the Run (including any data processing data) will be automatically backed up at the end of the Run (to a storage location pre-determined by an Administrator. See Appendix, Section 4).

   - This opens the Run Wizard's eighth window: Run comments (Figure 27).

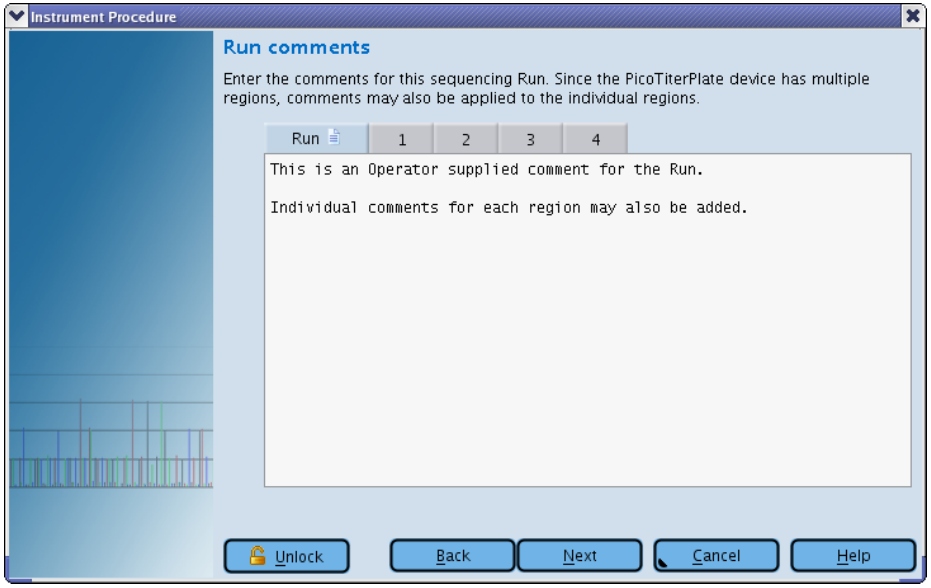


Figure 27: The Run Wizard's eighth window: Run comments

10. Enter any comments about the Run, and click Next.

    - This opens the ninth window: Confirmations (Figure 28)

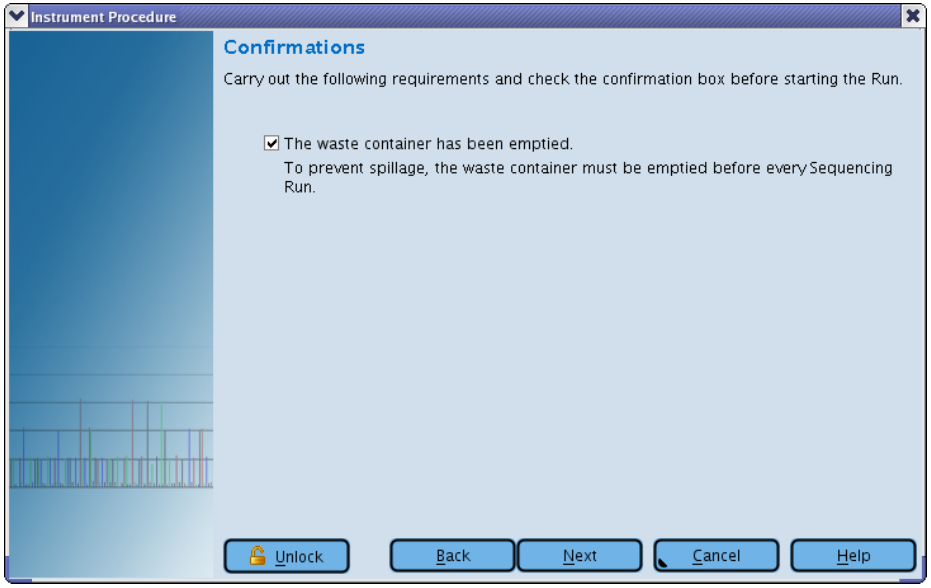


Figure 28: The Run Wizard's ninth window: Confirmations

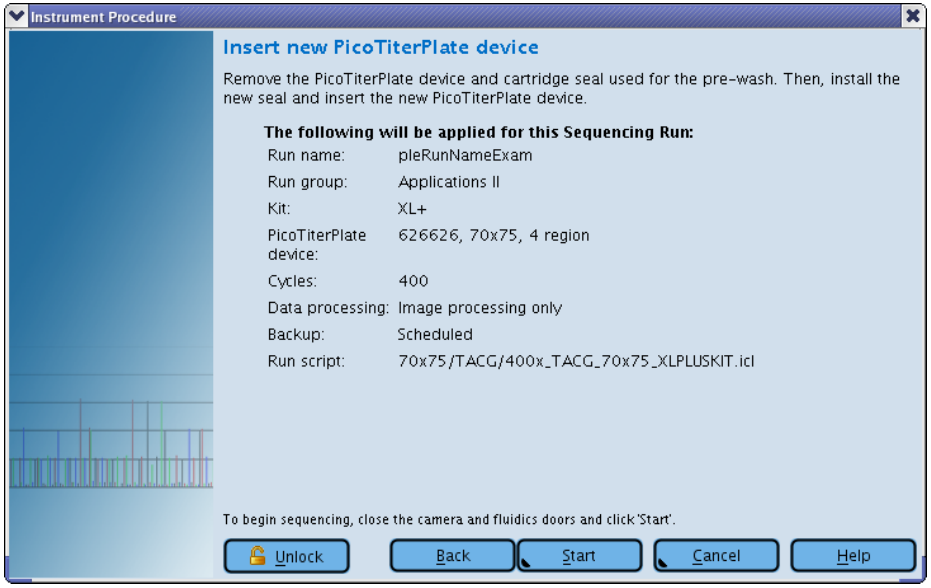


Figure 29: The Run Wizard's tenth, and last window: Insert new PicoTiterPlate device

11. Empty the waste container and check the box, then click Next.
    - This opens the tenth and last window: Insert new PicoPiterPlate device (Figure 29).

Step synchronization: Return to the bead deposition procedure for the PPiase Beads layer (Bead Layer 4, Section 3.2.5.5).

3.3.5 Insert the PicoTiterPlate Device and Launch the Sequencing Run

1. Wet a clean Kimwipe with 10% Tween-20 and wipe the surface of the cartridge.
2. Install the cartridge seal as described below:
    a. Make sure that the square ridge on the seal is facing up (see Figure 30**A**), and drop the seal in the cartridge groove (Figure 30**B**).
    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.

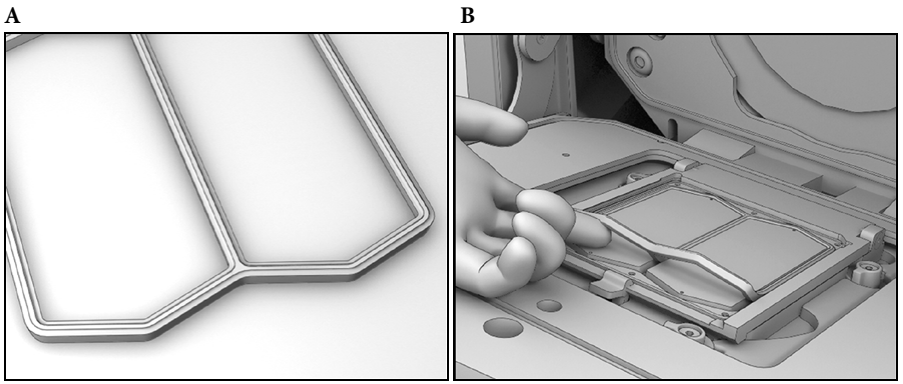


Figure 30: Orientation and placement of the cartridge seal

3. Press the PTP cartridge spring latch (Figure 31**A**) to lift the PTP frame from the cartridge.
4. When centrifugation of the Bead Layer 4 is complete (from Section 3.2.5.5, Step 7), remove the BDD from the centrifuge, and discard the BDD port seals that cover the loading ports and air vents.
5. 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.
6. 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.
7. 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 31**B**).
8. Close the PTP frame, making sure it is properly secured by the latch (Figure 31**C**).
9. 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.
10. Use a new Zeiss pre-moistened cleaning tissue to gently wipe the camera faceplate.
11. Allow the camera faceplate to air dry completely.
12. Close the camera door (Figure 31**D**).

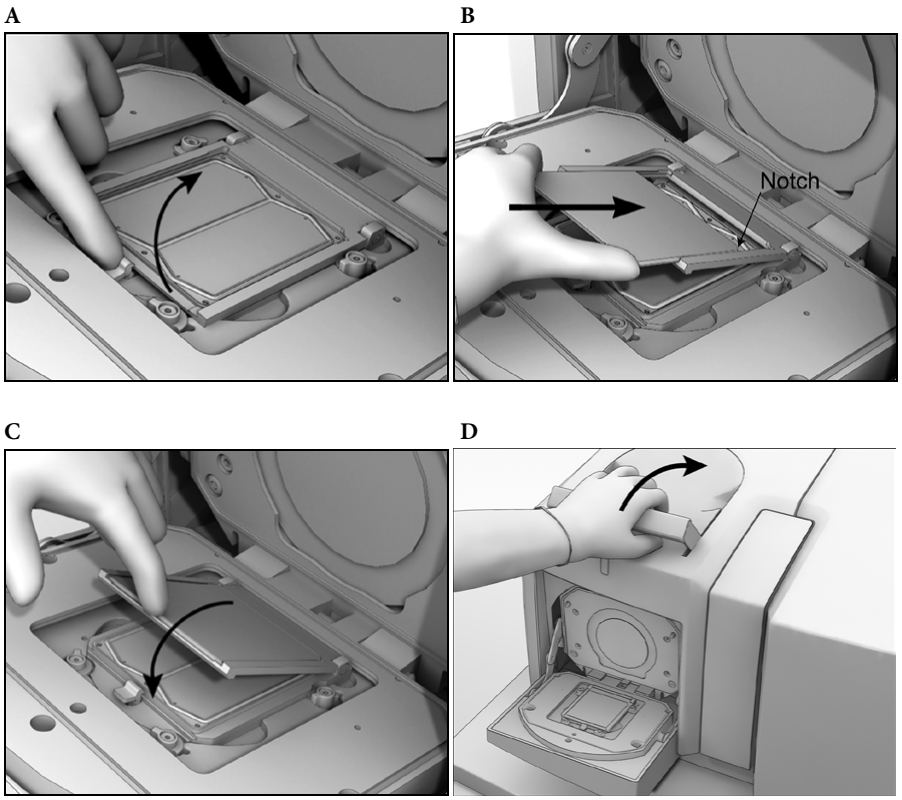


Figure 31: Loading a PTP device into the PTP frame, in the cartridge

13. Click the Start button in the Run Wizard's Insert new PicoTiterPlate device window (Figure 29) 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 32).
    - 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.

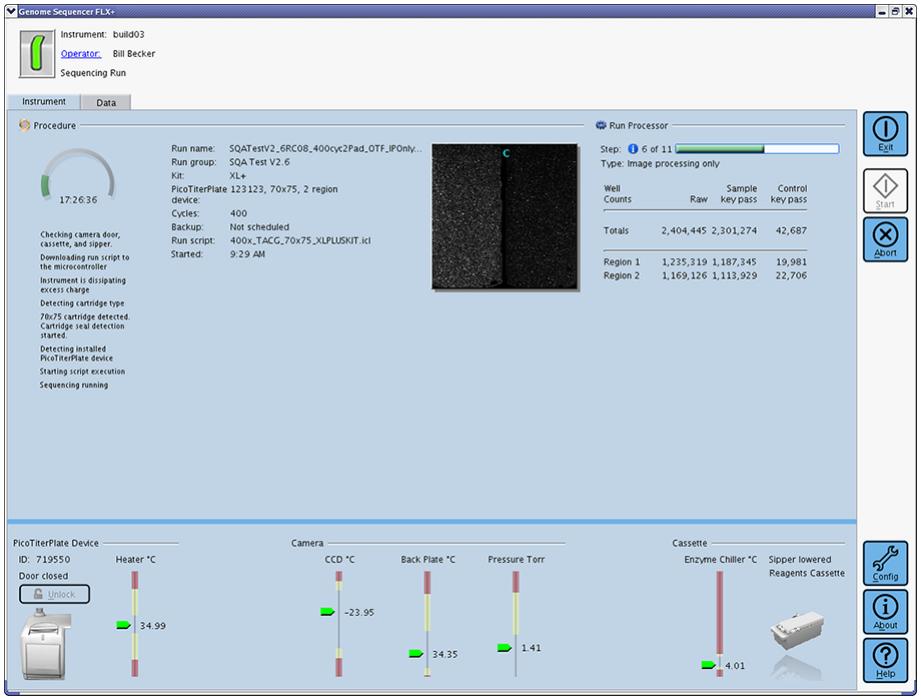


Figure 32: The Instrument tab of the GS Sequencer application main window during a sequencing Run

- 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 problem situations, 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:
    a. Close the GS Sequencer application
    b. Double-click the *systemStart* icon, located on the desktop
    c. 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 32), 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.

14. 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 very highly recommended to setup a network data backup mechanism to store sequencing Run data, because the data sets created by 400 cycle XL+ Runs are large, and the storage capacity on the instrument is limited. In addition, data processing on the instrument would take a substantial amount of time (up to a week or more).

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 *454 Sequencing 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 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 data processing option (assuming the largest bead loading regions available on the PTP device).

Note that the space required to perform these runs is smaller than it would have with previous versions of the 454 Sequencing System software. Version 2.6 of the software introduced image compression that allows a 40% reduction in space required to store the raw data.

Sequencing Kit	Number of Cycles	Raw Images Only	Raw Images Plus Image Processing	Raw Images Plus Full Processing
XL+	400	28-35 GB	45 GB	60 GB

Table 13: Approximate amount of data generated by the XL+ sequencing kit for the 400 cycle Run script and for each data processing option. These numbers assume that the 2 regions gasket is used.

Table of Contents

Published by

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

(1) 0511