BluePippin™ User Guide

High-Pass™ DNA Size Selection

of SMRTbell™ templates
for PacBio® *RS* Sequencing

Sage Science Part Nos:

PAC-20KB

PAC-40KB

sage science

Library Size Selection Chart
for PacBio® SMRTbell™ Templates

Updated June 16, 2015

Cassette Kit PN	System	High Pass Threshold Range	Cassette Definiton	PacBio Application	Validated by PacBio?	
PAC20KB	BluePippin	4-10kb	0.75% DF Marker S1 high-pass 4-10kb vs2	~20kb SMRTbell Templates	yes	1,2
PAC20KB	BluePippin	6-10kb	0.75% DF Marker S1 high-pass 6-10kb vs3	~20kb SMRTbell Templates	yes	
PAC20KB	BluePippin	15-20kb	0.75% DF Marker S1 high-pass 15-20kb	~20kb SMRTbell Templates	yes	
PAC40KB	BluePippin	30-40kb	0.75% DF Marker U1 high-pass 30-40kb		pending	3
BLF7510	BluePippin	NA	0.75% DF 2- 6 kb Marker S1	Iso-Seq	yes	
HTC7510	PippinHT	6-10kb	0.75% Agarose, 6-10kb High Pass, 75D	~20kb SMRTbell Templates	pending	4
HTC7610	PippinHT	15-20kb	0.75% Agarose, 15-20kb High Pass, 75E	~20kb SMRTbell Templates	pending	
HTC7710	PippinHT	30-40kb	0.75% Agarose, 30-40kb High Pass, 75F	* July 1, 2015 release		
ELD2010	SageELF	NA	0.75% 1 kb – 18 kb	Iso-Seq	yes	

Notes:

1 PAC20KB is the same kit as Sage PN BLF7510, except for the user quick guide.

2 The version2, 4-10kb, size selection accuracy can be affected by the DNA input amount. New users should use version 3, 6-10kb.

3 PAC40KB is the same kit as Sage PN BUF7510, except for the user quick guide.

4 The maximum input amount for the PippinHT is 1.5 ug. The PippinHT runs up to 22 samples per run.

Thank you for purchasing BluePippin High-Pass gel cassettes for size-selection of SMRTbell™ templates. The system is designed to collect DNA above a user-set threshold. Size-selection of SMRTbell templates prior to PacBio® **System Sequencing results in significantly longer subread lengths**.

Using this Guide

This guide is meant to provide a comprehensive workflow for using the BluePippin to size-select SMRTbell templates (prepared according to PacBio's protocol "Procedure & Checklist - SMRTbell™ Template Preparation Using BluePippin Size Selection" available for download on the Pacific Biosciences SMRT® community website at www.smrtcommunity.com/SampleNet/Sample-Prep.

 The instructions here provide special recommendations for software, hardware, and gel cassette usage for SMRTbell™ library preparations. However, users should read the **BluePippin Operations Manual** to completely familiarize themselves with the system and features, and safety and warnings.

Contents of this document

Installing pre-set protocols

Go to the BluePippin Support Page:
www.sagescience.com/product-support/bluepippin

BluePippin Support

Software Downloads

BluePippin Software v6.20, Cassette Definition Set 16
2.5MB

Pippin v.6.20 Software Release Notes CD16
171KB

1. Download the software update instructions.

Software Update Instructions
425KB

PacBio® SMRTbell™ Cassette Definition Set
2.5MB

2. Download the PacBio cassette definition set to a USB drive. Follow the software update instructions. This will install the protocols and update the software to the current revision.

Version 2 High Pass User Guide for20kb SMRTbell
951KB
Version 3 High Pass User Guide for20kb SMRTbell
1.4 MB

PacBio Users: Version 3 High-Pass Introduction and Best Practices
284KB

A. Preparing DNA samples

1. **Dilute up to 5 µg SMRTbell template into a final volume of 30 µl EB (PacBio Elution Buffer).**

    Note: Run 500 ng to 5 µg SMRTbell template per lane. We do not recommend running less than 500 ng per lane.

2. **Bring loading solution to room temperature and add 10 µl of loading solution to the 30 µl sample.**

    Note: Loading solution is viscous: pipet slowly to ensure complete transfer of the loading solution into the DNA sample.

3. **Mix throughly by gentle pipetting. Do not vortex. Spin briefely to collect the contents at the bottom of the tube.**

4. **Bring the DNA marker to room temperature.**

B. Creating a Protocol

The following instructions show how to program the size selection protocol for SMRTbell template preparation. Optional pre-programmed protocols may also be loaded onto a BluePippin , and are shown in the gray box on page 5.

1. **In the BluePippin Software, click the Protocol Editor Tab.**

2. **Press the "NEW" button, to program a new protocol.**

> For software steps, a small representation of the screen is provided, with a yellow circle that indicates the area of the screen that is being referred to in the text.


3. **Click "Cassette Type" folder icon, and select the appropriate cassette definition from the list below.**

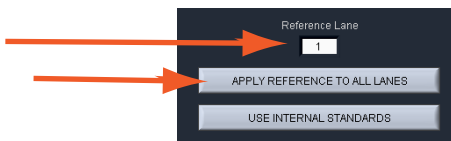| Cassette Kit PN	High Pass Threshold Range	Cassette Definiton
PAC20KB	6-10kb	0.75% DF Marker S1 high-pass 6-10kb vs3
PAC20KB	15-20kb	0.75% DF Marker S1 high-pass 15-20kb
PAC40KB	30-40kb	0.75% DF Marker U1 high-pass 30-40kb

4. **Select "Range" mode for the corresponding sample lanes to which the samples will be run**. Do not select a collection mode for the sample lane to which the DNA marker will be added.
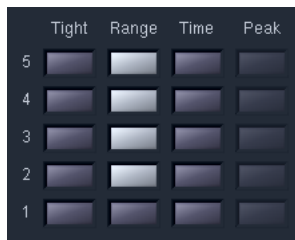
In this example, DNA reference marker will be loaded into lane 1, and the DNA samples will be loaded into lanes 2-5.

5. **Enter the lane number to which the DNA marker will be loaded into the "Reference Lane" field, and press the "APPLY REFERENCE TO ALL LANES" button**.

6. **Enter a base pair threshhold (BP Start) for DNA size selection, this value should be between 6000 and 40000.**

7. **Enter a value into the (BP End) field.** Enter 50000 or 80000, depending on the cassette kit being used:

Cassette Kit PN	High Pass Threshold Range	BP End Value
PAC20KB	6-10kb	50000
PAC20KB	15-20kb	50000
PAC40KB	30-40kb	80000

B.1 Loading optional pre-set protocols

The protocols may be downloaded from www.sagescience.com/support. Use the typical software update instructions. See page 2 for more information.

1. **Press the "LOAD" button.**

2. **Select the appropriate SMRTbell protocol from the protocol list.**

3. **The protocol names and settings are shown below.**

Protocol: **SMRTbell 20kb, 10000bp High-Pass v3**

	Tight	Range	Time	Peak	Ref Lane	BP Target	BP Start	BP End	BP Pause
5					1	30000	10000	50000	0
4					1	30000	10000	50000	0
3					1	30000	10000	50000	0
2					1	30000	10000	50000	0
1					1	0	0	0	0

8. **Enter Sample information into the "Sample ID" fields (optional).**
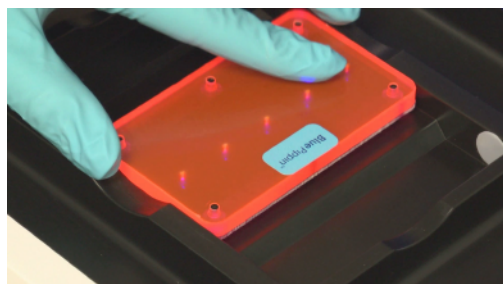

9. **Press "Save As" to name and save a new protocol.**

C. Calibrating the Optics with the Calibration Fixture

1. **In the BluePippin Software, click the Main Tab.**

2. **Place the calibration fixture onto the optical nest.** The dark side of the fixture must be down, and completely cover all LED detectors.

Correct placement of the calibration fixture.

3. **Close the Lid.**
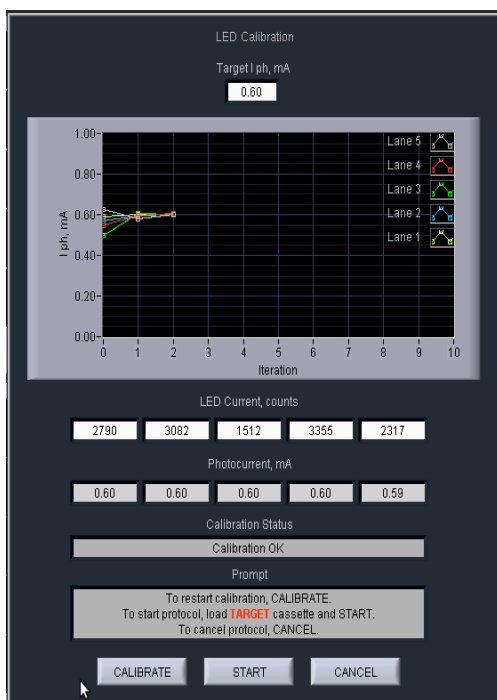
4. **Press the "CALIBRATE" button**.

5. **A calibration sub-window will launch.**

6. **Check that the calibration target box ("Target I ph, mA") at the top of the window reads "0.60".** If not, manually change the target value to "0.60".

7. **Press "CALIBRATE" button within the calibration window.**

The Calibration sub-window after a successful calibration. If the status window reads "Calibration OK", proceed to the next step.

Note: The optical calibration routine ensures that each LED light intensity is at the correct level (0.60 mA, in this case). The LEDs will adjust individual current (counts) values to achieve this intensity. If the count level is <200, the test will fail, likely due to light infiltration such as an open lid. The maximum count value is 4095, and if the LED cannot achieve the proper intensity at that level, the test will fail due to a faulty LED.

8. **Press "EXIT" to return to the Main Screen.**

D. Inspecting the Gel Cassette

1. **Remove the gel cassette from the foil packaging.**

2. **Inspect the levels of buffer in all buffer reservoirs.** Reservoirs should be nearly full. If the buffer level in any reservoir appears lower, or less than 50% full, fill with spare electrophoresis buffer (provided with the BluePippin reagent kit).


If a buffer reservoir is low, fill with spare buffer.

3. **Inspect for bubbles due to delamination of agarose from the bottom of the cassette in the region used for optical detection of DNA.** To inspect, turn the cassette upside down and gently tilt the cassette under a light source. If a bubble is detected, do not use the affected lane to run the DNA reference marker. Affected lanes can be used for size selection of sample DNA.
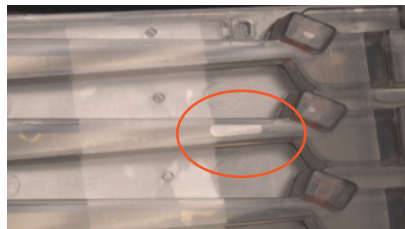

If the agarose has delaminated in the optical region, do not use that lane to run the reference marker .

E. Preparing the Cassette for Loading
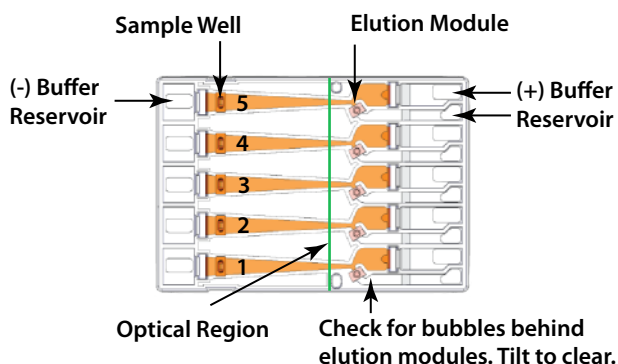
1. D**islodge air bubbles from behind the elution wells.** Tilt the cassette sample well side down, and tap gently to release the bubbles.

2. **Place the cassette into the optical nest.** Keep the cassette slightly tilted down so that the air bubbles do not return to the area behind the elution modules. Make sure the cassette is fully seated into the bottom of the nest to ensure proper optical alignment.

3. **Remove the white tabbed adhesive strips from the cassette.** Place one hand on the cassette, and hold it firmly in the nest. Grab the white tabs and pull the strips firmly and slowly toward the front of the instrument until they are removed.

4. **Completely remove all buffer from each elution module, and replace with 40 µl of fresh electrophoresis buffer** (provided with the BluePippin reagent kit). When refilling, dispense the fresh buffer slowly, raising the pipette tip slowly from the bottom of the elution module, to avoid trapping air bubbles in the module.

5. **Re-seal the elution wells with an adhesive tape strip (supplied with cassette packaging)**. Place the tape over the elution wells and rub firmly to seal the elution ports. The port should be tightly sealed without any wrinkles around the edges of the port.
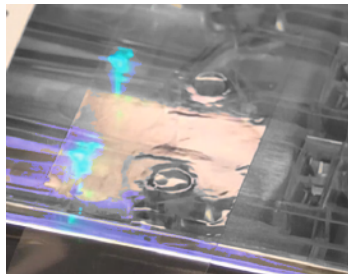
Rub firmly to ensure a tightly sealed elution port.

6. **Close the lid.**

7. **Perform the Continuity Test.** Press the "Test" button. The test sub-window will open, and the test routine will automatically measure the current in each separation and elution channel. A successful continuity test will return a "PASS" indication. The cassette temperature must be above $17^{O}C$ ($62^{O}F$). If a lane fails due to low elution current (the affected lane will be highlighted with an orange color), refilling the elution module (see step 4 above) will usually resolve the problem.
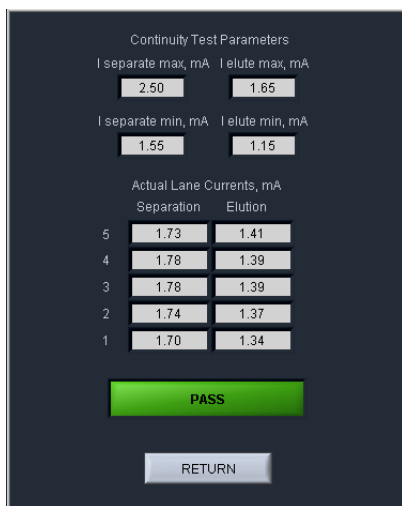
A successful continuity test will return a "PASS" indication.

8. **Press "Return" to close the Continuity Test sub-window.**

F. Loading Samples

buffer overlay
sample
field lines

proper loading geometry

bad loading geometry
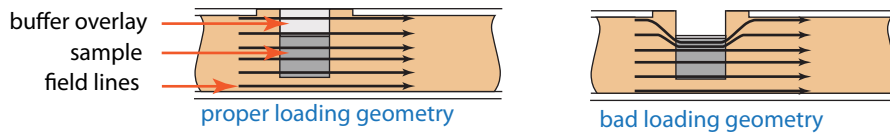
1. **Check the buffer levels in the sample wells**. Make sure that sample wells are completely full to the top with electrophoresis buffer. Fill with additional buffer if necessary.

2. **Remove 40μl of buffer from the first sample well, and load 40μl of sample or marker into that well.** Take care not to pierce the agarose with the pipette tip. There is gel on all sides and at the bottom of the sample well. In addition, there is an agarose "chimney" surrounding the top of the sample well that protrudes up through the cassette cover. When removing buffer, some users find it useful to immerse the pipette tip just below the surface of the buffer and follow the liquid level down with the tip as the buffer is removed. When buffer removal is completed, there will be ~30μl of buffer left in the well. When adding sample, place the tip of the pipette just below the surface of the buffer, and slowly eject the sample. Don't be concerned if the sample well slightly overfills. Then density of the sample will allow it to sink before it can flow out of the well.

3. **Repeat step 2 for the remaining four wells.**

Note: Cassettes may be reused if 2-3 lanes are used (1-2 samples). Reseal the cassete with the adhesive tape provided and store at RT. However one remaining lane must again be loaded with the external marker for calibration.

G. Running

1. **Close the lid, go to the Main Tab, and make sure the proper protocol is loaded in the "Protocol Name" field.**

2. **Press "START".** The run will automatically stop when every collection is complete. Electrogram images of the DNA markers in the Mains screen should appear as shown below.
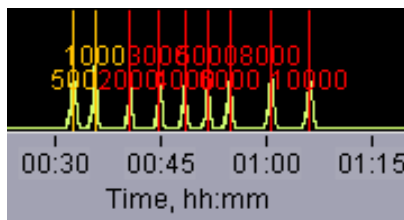

Marker S1
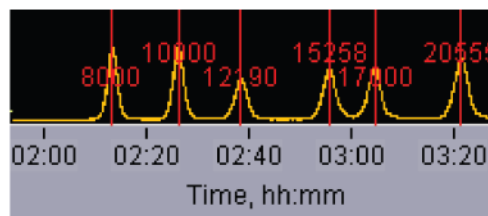
Marker U1

Approx run times: 2-3 hours

H. Collecting Fractions

1. **For best recovery, wait at least 30-45 minutes after end of run before removing samples from the elution. modules.**

2. **Remove samples using a standard 100-200μl pippette.** Aspirate slowly to minimize breakage of large DNA fragments. Samples will be in a Tris-TAPS buffer at a volume of approximately 40μl.

3. **Optional: Additional 10-30% yield can often be achieved by rinsing the elution well with TE+0.1% Tween 20.** Tween solution is provided by Sage Science with cassette kits for HMW collections.

   - Add 40μl Tween solution (supplied with the cassettes) to the elution well
   - Wait 1 minute
   - Remove solution and pool with the original extracted sample, or process separately

4. **Remove the cassette and dispose of properly.** Do not keep used cassettes in the Pippin with the cover closed. Humidity from the cassette may cause damage to the electrodes.
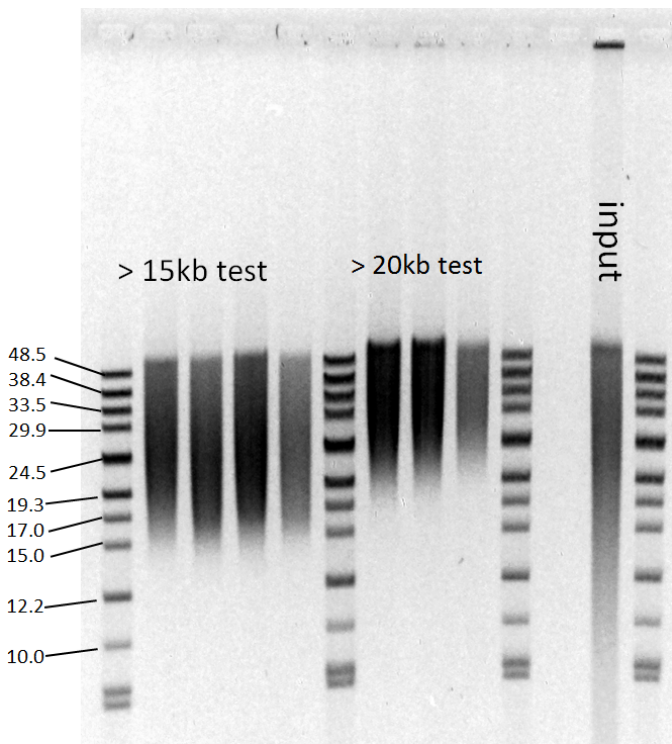
I. Gel Analysis Images of High-Pass Collections

The following pages show analytical gel images of sheared samples after size selection with High Pass prtocols. Gels were run using the Pippin Pulse pulsed-field electrophoresis power supply (PPI0200).
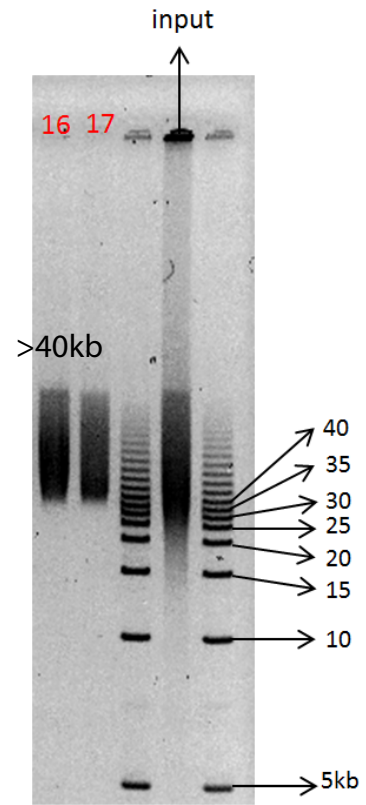
Programmed 6 kbp Start
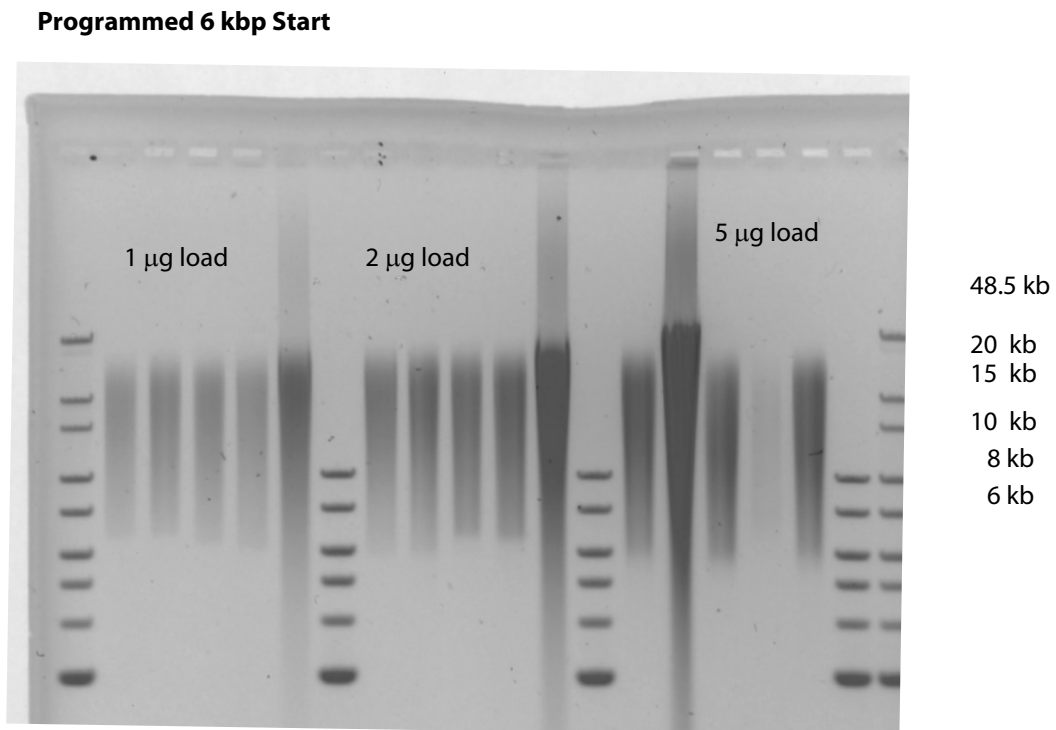
Programmed 10 kbp Start
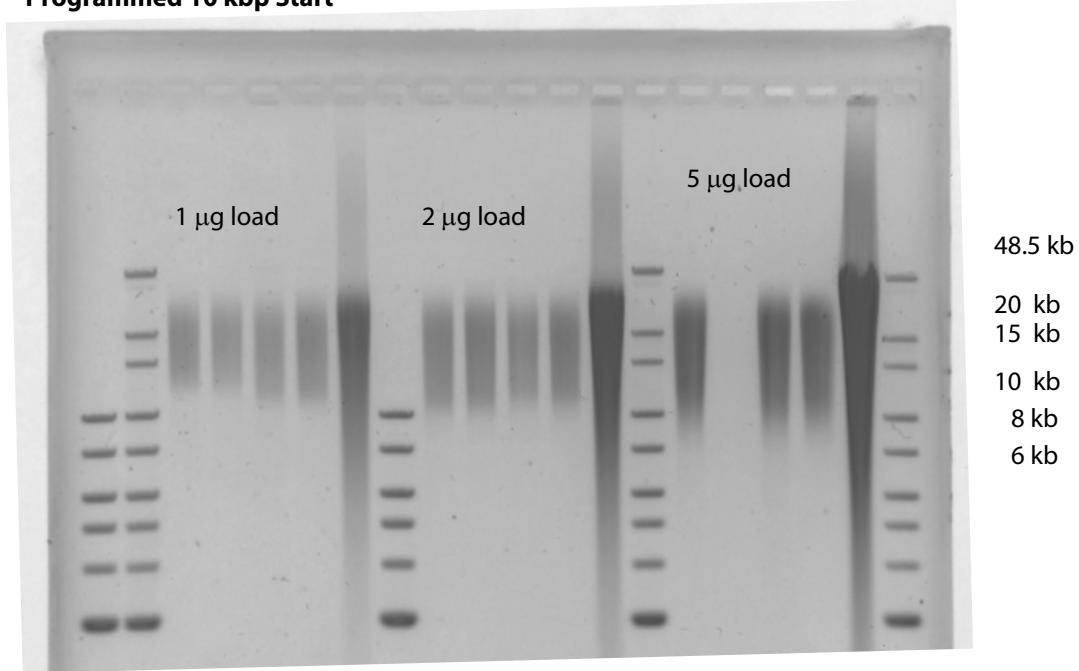
**Programmed 15 and 20 kbp Start (5 mg input)**

Programmed 40 kbp Start (5 mg input)

J. DNA Control for High-Pass Collections

High Pass control DNA (Product No. CHP-7504) can be used to evaluate whether the High Pass protocol is working as expected. The control DNA is a ladder containing fragments ranging from 0.5kb – 48kb, formulated to load 1.25μg of DNA per sample lane.

Control DNA collected with 6,7, 8 and 10kbp High Pass settings
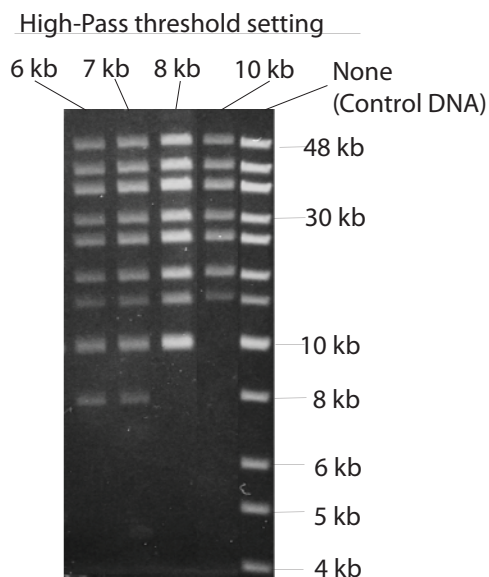
Control DNA collected with 20kbp High Pass settings