10 kb to 20 kb Template Preparation and Sequencing with Low-Input DNA

Before You Begin

To perform this procedure, you must have the PacBio®:

- DNA Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit
- DNA Sequencing Kit
- DNA Control Complex
- SMRT® Cells for standard sequencing

This procedure can be used to prepare 10-20 kb libraries from 50 ng up to 200 ng of sheared and concentrated DNA, or at least 100 ng into shearing. Note: for input amounts between 200 ng and 1 µg, the standard 10 kb library prep protocol may be used.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	DNA Damage Repair
10 to 20 kb	8 kb to 22 kb	50 to 200 ng	Blunt	Required

Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear your DNA sample, following the g-TUBE user manual available for download from the Covaris website, with one change -- reduce the sample volume from 150 µL to **50 µL**.

Note: After the first spin, make sure that all of the sample has passed into the lower chamber. If any sample remains, re-spin. If necessary, add 10 µL EB or TE to the upper chamber, flick the g-TUBE or pipette up and down several times, and spin again. Depending upon the quality of your sample, approximately 20% to 50% sample loss is to be expected as a result of the shearing and concentration process.

STEP	✓	Concentrate DNA	Notes
1		Add **0.5X** volume of AMPure® PB magnetic beads. _____ µL of sample X **0.5X** = _____ µL of beads Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process. Consistent and efficient recovery of your sample is critical to successful SMRTbell™ template preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Using the DNA shearing methods and subsequent AMPure PB bead purification steps described below, you should recover approximately 50%-80% of your input DNA (by mass). Typical yields, from pre-purified DNA (where smaller fragments are already eliminated as a result of the shearing process) are between 80-100%.	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by mixing in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After mixing, the bead/DNA mixture should appear homogenous. We recommend using a VWR vortex mixer with a foam microtube attachment (see the *Guide's* Overview section for part numbers). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this Procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	

STEP	✓	Concentrate DNA	Notes
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.	
13		Add **37 µL** of Pacific Biosciences' Elution Buffer to the beads to elute the DNA. – Mix until homogeneous. – Vortex for 1-2 minutes at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Proceed to the next step, if possible. If necessary, store at -20°C to continue later.	

Repair DNA Damage

Use the following table to repair any DNA damage.

1. In a LoBind microcentrifuge tube, add the reagents below.

Note: premix damage repair buffer, NAD+, ATP high, and dNTPs if you are preparing more than 1 sample.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	–		37 µL	–		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
DNA Damage Repair Mix			2.0 µL			
Total Volume			50.0 µL	–		

*To determine the correct amount of H_2O to add, use your actual DNA amount noted in the Notes column.

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 20 minutes, then return the reaction to 4ºC for 1 minute.

Repair Ends

Use the following table to prepare your reaction then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		50 µL	–		
End Repair Mix		20 X	2.0 µL	1X		
Total Volume			52.0 µL	–		

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes (no longer), return the reaction to 4ºC.

STEP	✓	Purify DNA	Notes
1		Add **0.5X** volume of AMPure PB beads to the End-Repair reaction. (For detailed instructions on AMPure PB bead purification, see the Concentrate DNA section).	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **32-33 µL** Elution Buffer.: – Mix until homogeneous. – Vortex for 1-2 minutes at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Proceed to the next step if possible. If necessary, store at -20°C to continue later.	

Prepare Blunt-Ligation Reaction

Use the following table to prepare your blunt-ligation reaction:

1. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts. Add the adapter to the well with the DNA. All other components, including the ligase, should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)			32 µl			
Annealed Blunt Adapter (20 µM)		20 µM	1.0 µL	0.5 µM		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
Total Volume	–	–	40.0 µL	–		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 25ºC for 45 minutes.
5. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonucleases after this step.

Add exonucleases to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				40 µL
Mix reaction well by pipetting				
ExoIII		100.0 U/µL		0.5 µL
ExoVII		10.0 U/µL		0.5 µL
Total Volume				41 µL

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37ºC for 45 minutes, then return the reaction to 4ºC. Do not exceed 1 hour incubation time. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 final purification steps. The first uses **0.5X** volumes of AMPure PB beads, followed by purification with **0.45X** volumes of AMPure PB beads.

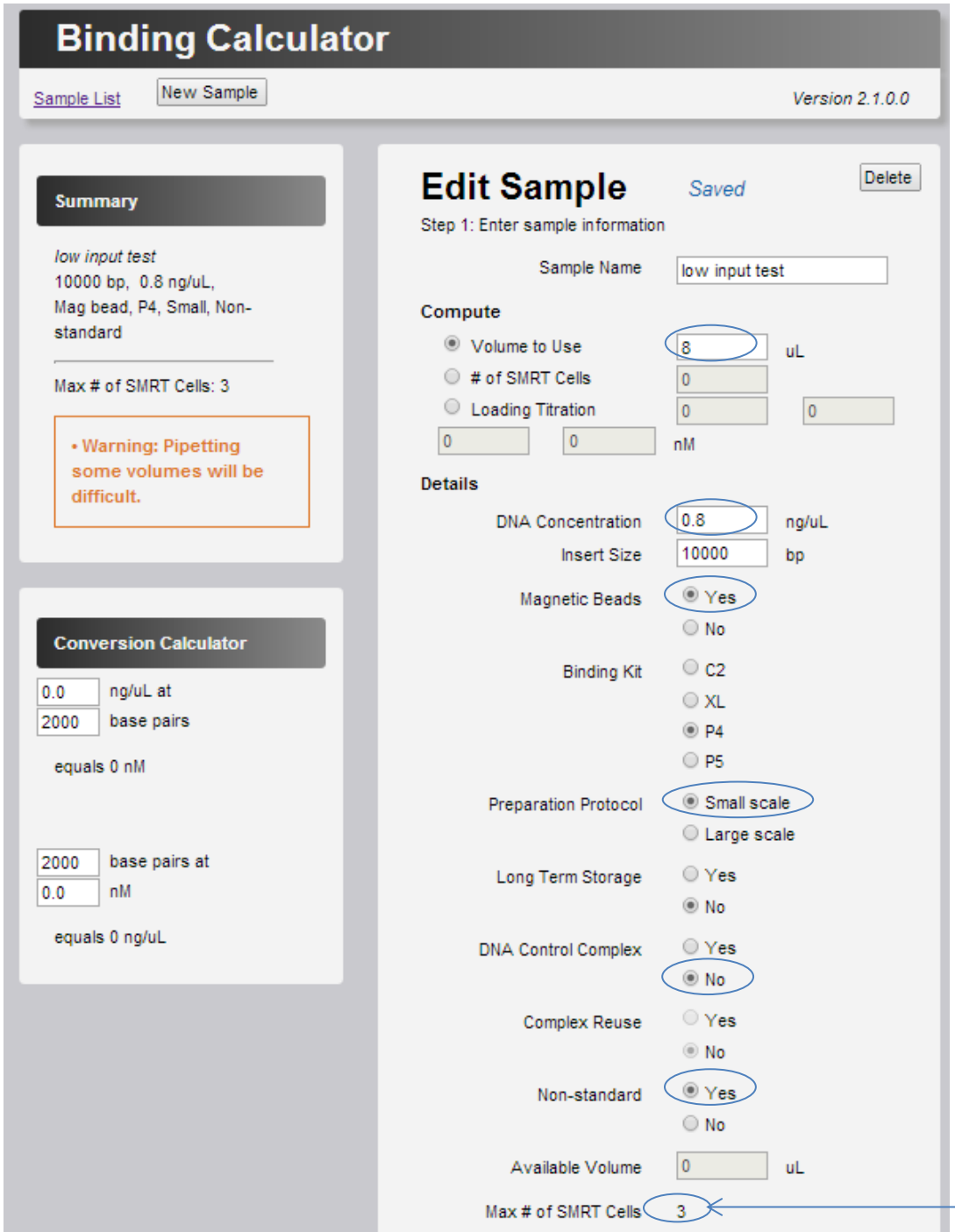
STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add **0.5X** volume of AMPure PB beads to the exonuclease-treated reaction. (For detailed instructions on AMPure PB bead purification, see the Concentrate DNA section).	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **50 µL** of Elution Buffer. Mix for 10 minute at 2000 rpm: – Mix until homogeneous. – Vortex for 1-2 minutes at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add **0.45x volume** of AMPure PB beads to the **50 µL** of eluted DNA.	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **8-10 µL** of Elution Buffer: – Mix until homogeneous. – Vortex for 1-2 minutes at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Check quantitation with the Qubit® dsDNA HS Assay Kit. If there is too little sample, estimate concentration based on 10% yield of input amount into damage repair.	

Anneal and Bind SMRTbell™ Templates

You must have a PacBio DNA Polymerase Binding Kit for this step. To anneal sequencing primer and bind polymerase to SMRTbell templates, follow the Calculator recommendations with the following set-up:

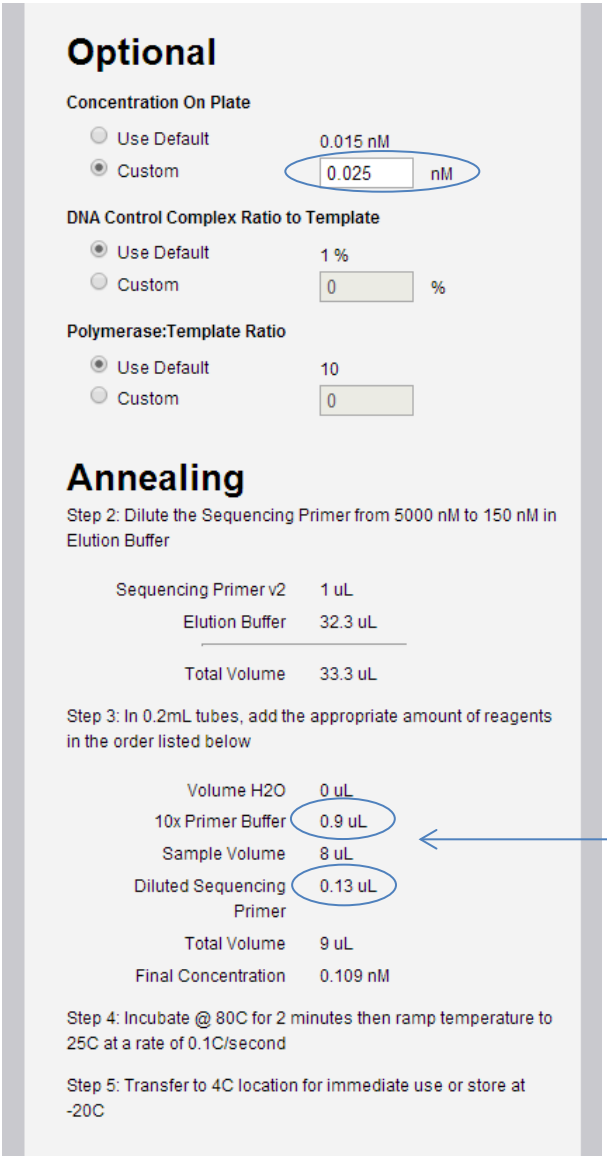
1. Under **Edit Sample**, enter the Volume to Use and DNA Concentration (measured or estimated - assuming 10% library yield). Then select the following:
   - Magnetic Beads: Yes
   - Preparation Protocol: Small scale
   - DNA Control Complex: No
   - Non-standard: Yes



Note the suggested number of SMRT Cells

2. Under **Optional**, enter a Custom Concentration on Plate:
   -For P4 polymerase, enter 0.025 nM
   -For P5 polymerase, enter 0.040 nM

3. Under **Annealing,** pre-mix 10x Primer Buffer and Diluted Sequencing Primer at higher volumes to eliminate small volume pipetting



Optional

Concentration On Plate

Use Default	0.015 nM	
Custom	0.025	nM

DNA Control Complex Ratio to Template

Use Default	1 %	
Custom	0	%

Polymerase:Template Ratio

Use Default	10	
Custom	0	

Annealing

Step 2: Dilute the Sequencing Primer from 5000 nM to 150 nM in Elution Buffer

Sequencing Primer v2	1 uL
Elution Buffer	32.3 uL
Total Volume	33.3 uL

Step 3: In 0.2mL tubes, add the appropriate amount of reagents in the order listed below

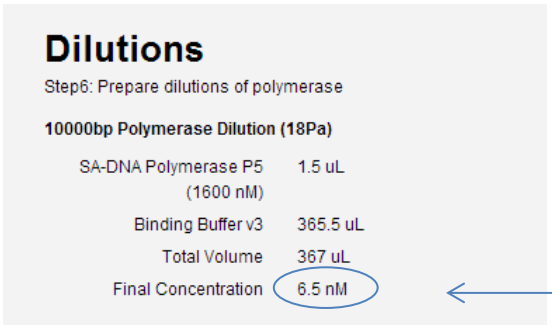
Volume H2O	0 uL
10x Primer Buffer	0.9 uL
Sample Volume	8 uL
Diluted Sequencing Primer	0.13 uL
Total Volume	9 uL
Final Concentration	0.109 nM

Step 4: Incubate @ 80C for 2 minutes then ramp temperature to 25C at a rate of 0.1C/second

Step 5: Transfer to 4C location for immediate use or store at -20C

a. Prepare a 10x pre-mix by combining 9.0 µL 10x Primer Buffer and 1.3 µL Diluted Sequencing Primer
b. Add 1.03 µL pre-mix to the sample

4. **Dilutions.** If polymerase is diluted to <8.0 nM, increase the polymerase:template ratio until 8.0 nM final concentration is obtained (or simply dilute polymerase 1/200 in Binding Buffer v3):



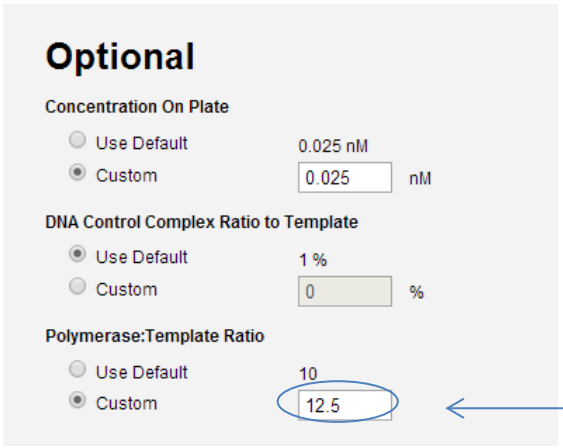
Dilutions

Step6: Prepare dilutions of polymerase

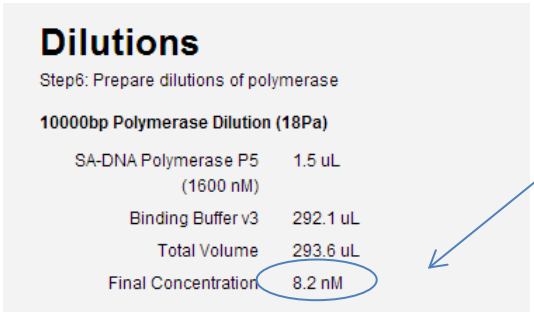
10000bp Polymerase Dilution (18Pa)

SA-DNA Polymerase P5 (1600 nM)	1.5 uL
Binding Buffer v3	365.5 uL
Total Volume	367 uL
Final Concentration	6.5 nM

If this number is <8.0 nM, see recommendation under “Optional” below.



Increase this number as required until the polymerase dilution is at least 8.0 nM (see below).



5. Use the entire complex to sequence the number of SMRT Cells recommended (by the calculator) in one run.

6. If desired, the bead-bound complex from sample plate can be used the next day by doing the following:
   -Pool the remaining beads from multiple SMRT Cells.
   -Add the Bead Binding Buffer to bring the volume to 19 µL (for 1 SMRT Cell), or the required volume for the closest number of SMRT Cells. For example, if 3 cells were run, and the remaining pooled volume is 26 µL, add 2 µL Bead Binding Buffer to bring the volume to 28 µL for 2 SMRT Cells. The required volume for any number of SMRT Cells may be determined with the binding calculator.

   The expected yield from reused beads depends on the amount of Bead Binding buffer added. If none is needed, it may be close to the original yield (if used the following day). The yield will decrease with longer times between runs.