# Procedure and Checklist - 20 kb Template Preparation Using BluePippin™ Size-Selection System

## Before You Begin

This procedure can be used to prepare size-selected libraries from 5 µg of sheared and concentrated DNA using the BluePippin Size-Selection system. If preparing larger amounts of DNA, scale all the reaction volumes proportionally (e.g., if the input amount of DNA is double the amount set forth in this procedure, then double all the reaction volumes listed in the tables). If a BluePippin Size-Selection system is not available, see the *Procedure & Checklist – Greater Than 10 kb Template Preparation Using AMPure® PB Beads*. Note that size selection using the AMPure PB beads does not completely remove all short SMRTbell templates. As a result, the average subread lengths may be shorter.

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 more than 80% of your input DNA (by mass) after AMPure PB bead purification.

| Insert Size Target | Insert Size Range | Sheared and Concentrated DNA Amount | Ligation | DNA Damage Repair |
|---|---|---|---|---|
| 10 kb to 20 kb | 15 kb to 20 kb(size-selected using BluePippin system) | 5 µg | Blunt | Required |

## Evaluate Genomic DNA Quality

We recommend using a Pulsed Field Electrophoresis system for evaluating gDNA quality. There are two commercially available systems capable of resolving HMW DNA fragments and smears.

- Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis system. The procedure is available here.
- Sage Science's Pippin Pulse Electrophoresis Power Supply. The procedure is available here.

Alternatively, Advanced Analytical Technologies, Inc. FEMTO Pulse is an automated pulsed-field capillary electrophoresis instrument for evaluating the integrity of genomic DNA with a run time of approximately 1.5 hours.

## Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear your DNA sample. General recommendations for use of g-TUBEs can be found in the g- TUBE user manual available for download from the Covaris website (see http://covarisinc.com/products/g-tube/). Note that although Covaris provides recommendations for shearing gDNA to 20 kb, these parameters result in sheared gDNA with average insert sizes of less than 20 kb, and are **NOT** suitable for use in this protocol. To generate sheared gDNA with average insert sizes >20 kb, you **MUST** follow the specific instructions provided here:

1. Dilute DNA to 200-300 ng/µl in Elution Buffer (EB). The sample volume may range from 20-100 µL.
2. Shear at 5500 rpm (2029 x g) for 2 minutes in an Eppendorf® MiniSpin plus.
3. Check for any residual sample remaining in the upper chamber. If present, re-spin for another 2 minutes. Continue spinning until all the sample has passed through the orifice.
4. Invert and spin at 5500 rpm (2029 x g) until all the sample has passed through the orifice.
5. Recover your sample into a 1.5 mL LoBind microcentrifuge tube.

Depending upon the quality of your sample, approximately 20% sample loss is to be expected as a result of the shearing and concentration process. Therefore, be sure to have sufficient amounts of starting DNA in order to have at least 5 µg of sheared and concentrated DNA (140 ng/µL) for the subsequent repair steps.


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| STEP | ✓ | Concentrate DNA | Notes |
|---|---|---|---|
| 1 | | Add **0.45X** volume of AMPure® PB magnetic beads.<br>_____ µL of sample X **0.45X** =_____µL of beads<br>Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature.<br>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. | |
| 2 | | Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix. | |
| 3 | | Quickly spin down the tube (for 1 second) to collect the beads. | |
| 4 | | Allow the DNA to bind to beads by gentle end-over-end rotation for 15-20 minutes at room temperature. We recommend using a tube rotator. | |
| 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. | |
| 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.<br>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.<br>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.<br>– Do not remove the tube from the magnetic rack.<br>– 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.<br>– Do not disturb the bead pellet.<br>– After 30 seconds, pipette and discard the 70% ethanol. | |
| 9 | | Repeat step 8 above. | |
| 10 | | Remove residual 70% ethanol.<br>– 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.<br>– Place the tube back on magnetic bead rack.<br>– Let beads separate fully.<br>– Pipette off any remaining 70% ethanol. | |
| 11 | | Check for any remaining droplets in the tube. If droplets are present, repeat step 10. | |

| STEP | ✓ | Concentrate DNA | Notes |
|---|---|---|---|
| **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** | | Calculate appropriate volume of Elution Buffer.<br>_____ ng X **0.5** / (____ng/µL) =_____µL of Elution Buffer needed<br>The minimum DNA concentration required to proceed to the next step (End-Repair) is **140 ng/µL** with preferred mass of at least **5 µg**. | |
| **14** | | Add the Pacific Biosciences® Elution Buffer volume (calculated in step 12 above) to your beads. Tap the tube with finger gently to mix, until beads are uniformly resuspended. Do not pipet to mix.<br>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.<br>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.<br>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube.<br>– Discard the beads. | |
| **15** | | Verify your DNA amount and concentration using a Qubit quantitation platform.<br>– Measure the DNA concentration using a Qubit fluorometer.<br>– Using 1 µL of the eluted sample, make a 1:10 dilution in EB.<br>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.<br>Yield up to this point should be **80%**. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulsed-field capillary electrophoresis. | |
| **16** | | The sheared DNA can be stored for up to 24 hours at 4ºC or at -20ºC for longer duration. | |
| **17** | | Actual recovery per µL and total available sample material: _____ | |

## ExoVII Pre-Treatment of DNA

Use the following table to remove single-stranded ends from 5 μg of sheared gDNA at 140 ng/ul. If starting with more than 5 μg of sheared gDNA, scale the reaction volumes proportionally (i.e., for a mass between 6-10 μg of DNA scale the total volume to 96 μL).

1. In a LoBind microcentrifuge tube, add the following reagents:

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| Sheared DNA (5 μg) | | | 36.0 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 | | |
| ExoVII |  | 10 U/μL | 1.0 μL | 0.2 U/μL | | |
| Total Volume | | | 48.0 μL | | | |

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 15 minutes, then return the reaction to 4ºC.

## Repair DNA Damage

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reactions volumes proportionally.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (ExoVII treated) | | | 48.0 µL | | | |
| DNA Damage Repair Mix |  | 25 X | 2.0 µL | 1X | | |
| Total Volume | | | 50.0 µL | | | |

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37ºC for 20-60 minutes, return the reaction to 4ºC for 1 to 5 minutes.

## Repair Ends

Use the following table to prepare your reaction then purify the DNA. For more than 5 µg input DNA, scale all reactions volumes proportionally.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (Damage Repaired) | | | 50.0 µL | | | |
| End Repair Mix |  | 20 X | 2.5 µL | 1X | | |
| Total Volume | | | 52.5 µL | | | |

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5-10 minutes, return the reaction to 4ºC. Proceed to the next step.

| STEP | ✓ | Purify DNA | Notes |
|---|---|---|---|
| 1 | | Add **0.45X** volume of AMPure PB beads to the End-Repair reaction. | |
| 2 | | Mix the bead/DNA solution thoroughly by gently tapping the tube. Do not pipet to mix. | |
| 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 gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator. | |
| 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.<br>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.<br>– Do not remove the tube from the magnetic rack.<br>– 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.<br>– Do not disturb the bead pellet.<br>– After 30 seconds, pipette and discard the 70% ethanol. | |
| 9 | | Repeat step 8 above. | |
| 10 | | Remove residual 70% ethanol.<br>– 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.<br>– Place the tube back on magnetic bead rack.<br>– 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 | | For 5 µg of input sheared gDNA, elute in **23 µL** Elution buffer. If you started with more than 5 µg input sheared gDNA, scale volume of EB proportionally (i.e., for 6-10 µg of DNA, elute in 46 µL EB).<br>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. Do not pipet to mix.<br>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.<br>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.<br>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.<br>– Discard the beads. | |

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| **14** | | Optional: Verify your DNA amount and concentration using a Qubit quantitation platform.<br>– Measure the DNA concentration using a Qubit fluorometer.<br>– Using 1 µL of the eluted sample, make a 1:10 dilution in EB.<br>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.<br>– The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis. | |
| **15** | | The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration. | |
| **16** | | Actual recovery per µL and total available sample material: _____ | |

## Prepare Blunt Ligation Reaction

Use the following table to prepare your reaction, adding the components below in the order listed. Be sure to mix insert gDNA and adapter BEFORE adding ligase. For more than 5 µg input DNA, scale all reaction volumes proportionally.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (End Repaired) | | | 23.0 µL | | | |
| Blunt Adapter (20 µM) | | 20 µM | 10.0* µL | 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 | | | |

*Note that this increase in adapter during ligation minimizes the incidence of chimeras. Adapter dimers are then efficiently removed during size selection in the BluePippin System. This is not recommended for libraries which are not being size selected using the BluePippin System.

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 15 – 60 minutes. Ligation reactions can be left at 25 ºC overnight, if desired.
4. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC.

## ExoIII/VII Digestion to Remove Failed Ligation Products

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

| Reagent | Tube Cap Color | Stock Conc. | ✓ | Volume |
|---|---|---|---|---|
| Ligated DNA | | | | 40.0 µL |
| Mix reaction well by pipetting | | | | |
| ExoIII | | 100.0 U/µL | | 1.0 µL |
| ExoVII | | 10.0 U/µL | | 1.0 µL |
| Total Volume | | | | 42.0 µL |

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC.
4. You must immediately proceed with AMPure PB bead purification after this step.

## Purify SMRTbell™ Templates with 0.45X AMPure PB Beads

| STEP | ✓ | Purify SMRTbell™ Templates | Notes |
|---|---|---|---|
| 1 | | Add **0.45X** volume of AMPure PB beads to the exonuclease-treated DNA. | |
| 2 | | Mix the bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix. | |
| 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 gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator. | |
| 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.<br>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.<br>– Do not remove the tube from the magnetic rack.<br>– 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.<br>– Do not disturb the bead pellet.<br>– After 30 seconds, pipette and discard the 70% ethanol. | |
| 9 | | Repeat step 8 above. | |
| 10 | | Remove residual 70% ethanol.<br>– 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.<br>– Place the tube back on magnetic bead rack.<br>– 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 | | For up to 10 µg input non-selected library, elute in **31 µL** Elution buffer. If you size select more than 10 µg of SMRTbell library, scale volume proportionally.<br>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. Do not pipet to mix.<br>– Elute the DNA by letting the mix stand at room temperature for 2 minutes<br>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.<br>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.<br>– Discard the beads. | |

| | | | |
|---|---|---|---|
| **14** | | Verify your DNA amount and concentration using a Qubit quantitation platform.<br>– Measure the DNA concentration using a Qubit fluorometer.<br>– Using 1 µL of the eluted sample, make a 1:10 dilution in EB.<br>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.<br>Yield up to this point should be 40-60%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis. | |
| **16** | | Proceed with size-selection after AMPure PB Bead purification of exonuclease-treated libraries. Otherwise, samples may be stored at -20ºC at this point. | |
| **17** | | Actual recovery per µL and total available sample material: _____ | |

## Size-Selection Using the BluePippin™ System

Follow the general instructions in the BluePippin User Manual and User Guides (see www.sagescience.com), and the specific recommendations below for >15 kb or >20 kb size selection of the SMRTbell templates. Before performing this procedure, ensure that your BluePippin software is up-to-date by checking Sage's website.

It is highly recommended to perform qualitative and quantitative analysis using pulsed-field gel electrophoresis or pulsed-field capillary electrophoresis before size selection. This allows you to choose the appropriate "BPstart" value for Blue Pippin size selection. Choosing the "BPstart" value cutoff without knowing size distribution of SMRTbell™ Templates might lead to significant sample loss.

If average library insert sizes as determined by pulsed-field gel electrophoresis are less than 15 kb, you may perform >6 kb to >10 kb size-selection using the "0.75%DF Marker S1 high-pass 6-10kb v3" cassette definition file instead. Refer to the High Pass User Guide for SMRTbell Templates available on Sage's website (see www.sagescience.com) for more information.

| STEP | ✓ | Prepare DNA Samples for Each Lane | Notes |
|---|---|---|---|
| **1** | | Prepare up to 5 µg SMRTbell templates in a final volume of 30 µL Elution Buffer for each lane. Size selection using this protocol can be aggressive and if not cautious, recovery may be impacted. | |
| **2** | | Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. For multiple lanes, scale volumes proportionally. The Loading Solution is viscous so pipet slowly to ensure complete transfer into the DNA sample.<br>– Mix by gentle pipetting; do not vortex.<br>– Spin briefly to collect the contents at the bottom of the tube. | |
| **3** | | Follow the manufacturer's recommendations to set up a run protocol.<br>– When setting up the run protocol, select the "0.75%DF Marker S1 high-pass 15-20kb" cassette definition file.<br>– Using the "Range" selection mode, enter the desired "BPstart" value of 15000 or 20000 bp. A "BP End" value of 50000 bp should automatically appear. Be sure to assign a marker lane. | |
| **4** | | Load samples and start the run. Be sure to include the S1 marker in the correct lane. Typical run times are ~5.5 hours. | |
| **5** | | To maximize recovery of eluted DNA, wait at least 30 minutes after the run terminates before removing the sample from the elution chamber.<br>– Collect the eluate into a 1.5 mL DNA LoBind tube.<br>– Wash elution well with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution, and add wash to eluted sample. Washing the elution well may increase yield 10-20%. | |

## Purify Size-Selected SMRTbell™ Templates with 1X AMPure PB Beads

| STEP | ✓ | Purify DNA | Notes |
|---|---|---|---|
| 1 | | Add **1X** volume of AMPure PB beads to the size selected library. | |
| 2 | | Mix the bead/DNA solution thoroughly by gently tapping the tube. Do not pipet to mix. | |
| 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 gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator. | |
| 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.<br>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.<br>– Do not remove the tube from the magnetic rack.<br>– 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.<br>– Do not disturb the bead pellet.<br>– After 30 seconds, pipette and discard the 70% ethanol. | |
| 9 | | Repeat step 8 above. | |
| 10 | | Remove residual 70% ethanol.<br>– 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.<br>– Place the tube back on magnetic bead rack.<br>– 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** | | For up to 5 µg size-selected library, elute in **10 µL** Elution buffer.<br>For more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 20 µL EB).<br>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. Do not pipet to mix.<br>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.<br>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.<br>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.<br>– Discard the beads. | |
| **14** | | Verify your DNA amount and concentration using a Qubit quantitation platform.<br>– Using 1 µL of the purified sample, make a 1:10 dilution in EB.<br>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.<br>The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis | |
| **15** | | AMPure PB bead purified, size-selected libraries may be stored at -20C. | |

## Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

Before adding the primer to the SMRTbell template, pre-condition the primer by heating to 80ºC for 2 minutes, then place immediately on ice. (Note that if kept on ice during use, and stored at -20ºC, pre-conditioned primer may be used multiple times without re-heating.)

**RSII System**:
For the RSII System, follow the RSII Binding Calculator. Anneal 20X sequencing primer at a template concentration of 0.833 nM and incubate at 20ºC for 30 minutes.

Bind 10X P6 polymerase at an annealed template concentration of 0.500 nM (according to the Binding Calculator). For polymerase binding, incubation at 30ºC for 30 minutes is sufficient.

For more information about using the Binding Calculator, see the Pacific Biosciences *Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations*.

**Sequel System**:
For Sequel Systems, follow the SMRT Link Sample Setup instructions. Anneal 10X sequencing primer at a template concentration of 0.833nM and incubate at 20ºC for 60 minutes.

Bind 10X Sequel polymerase at an annealed template concentration of 0.500 nM (per SMRT Link Sample Setup). For polymerase binding, incubation at 30ºC for 4 hours is required.

## Prepare for Sequencing

**PacBio RSII System**:

For the RSII System, MagBead loading is required. Optimal loading of ~20 kb SMRTbell libraries using P6 polymerase can typically be achieved using an on-plate concentration of 100-125 pM. We recommend you perform an initial loading titration in this range to determine optimal loading for your sample.
For efficient binding to MagBeads, bound complexes (at 0.500 nM concentration) must be diluted in the appropriate ratio of MagBead Binding Buffer and MagBead Wash Buffer. Follow the Binding Calculator instructions to dilute your sample for MagBead binding.

**Sequel System**:
For the Sequel System, MagBead loading is required. Refer to SMRT Link Sample Setup for optimal loading recommendations.

For Sequel Systems, it is highly recommended to purify the complex using SMRTbell Clean Up Columns to remove excess primers and polymerase prior to sequencing. See the PacBio *Procedure & Checklist - Sample Purification Using SMRTbell Clean Up Columns v2 for MagBead Loading*.

Follow the SMRT Link Sample Setup instructions to anneal, bind and clean-up your samples.