# Procedure & Checklist
# >20 kb Template Preparation Using BluePippin™ Size-Selection System (15 - 20 kb Cutoff) for Sequel™ Systems

## Before You Begin

To perform this procedure, you must have the PacBio® Template Prep Kit and have reviewed the *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates*.

This procedure can be used to prepare size-selected libraries from 5 µg of sheared DNA using a 15 - 20 kb cut-off in the BluePippin Size-Selection system. It's important to note that when the "0.75%, DF Marker S1 high-pass 15 kb – 20 kb" protocol size-selection cutoff is set to 15 kb up to 20 kb, this defines the lower edge of the range to be collected.

Only high-quality, high molecular weight genomic DNA (gDNA) may be used for producing >20 kb libraries. To ensure success, gDNA size and integrity must be verified by pulsed field gel electrophoresis before beginning library preparation.

## DNA Handling

When constructing large insert libraries, it is highly recommended to perform gentle mixing instead of vortexing. Vigorous vortexing can induce damage to large fragments. Gentle mixing can be done on a rotator (same rotator used for MagBead binding) at room temperature for 30 minutes.

## Fragment and Concentrate DNA

Before performing large-scale shears, we highly recommend performing test shears to determine the best shearing condition to target distribution mode larger than the 17 kb marker when run on the Bioanalyzer system The following procedure can be used for a total mass of 4 µg – 30 µg in 150 µL per g-TUBE.

| STEP | | DNA Shearing | Notes |
|---|---|---|---|
| **1** | | Add 150 µL of sample in the g-TUBE. | |
| **2** | | Spin at 5600 (between 5000 – 6000) rpm for 2 minutes in an Eppendorf MiniSpin plus. Other centrifuges may be used but ensure to adjust the spin speed (rpm) based on the rotor size of the centrifuge. | |
| **3** | | Check for residual volume remaining in the upper chamber. If present, spin again at 5600 rpm for an additional two minutes. Repeat this spin cycle until most, if not all, of the sample has passed through the orifice. If a small volume persists, perform a final spin at 8000 - 10000 rpm to push everything through the orifice. Do not do this step if a large volume of the sample is still present in the upper chamber. A pipette can also be used to remove the residual volume that does not make it through the orifice. | |
| **4** | | Invert the g-TUBE and spin at 5600 rpm until all samples have passed through the orifice. Continue spinning the g-TUBE until the sample has passed through the orifice. | |
| **5** | | Repeat steps 2 to 4. | |
| **6** | | Recover the sample into a 1.5 or 2.0 mL LoBind microcentrifuge tube. | |

| 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 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 the bead/DNA solution by tapping the tube gently. | |
| **3** | | Quickly spin down the tube (for 1 second) to collect the beads. | |
| **4** | | Allow the DNA to bind to beads by gently rotating the tube for 30 minutes at room temperature. Ensure that the bead/sample mixture is mixing throughout the rotation process. | |
| **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.<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>– 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 13 above) to the beads and rotate at room temperature for 20 minutes.<br>– Mix by tapping the tube gently until the beads are suspended in solution.<br>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.<br>– Perform concentration measurements. Verify your DNA concentration using a Nanodrop® or Qubit® quantitation platform. If the DNA concentration is estimated to be equal to or below **12 ng/µL**, a Qubit system reading is required. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system.<br>– Discard the beads. | |
| **15** | | Perform qualitative and quantitative analysis using a Bioanalyzer® instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used.<br>Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent Technologies' guides for specific information on the range of the specific kit you might be using.<br>Note that typical yield, at this point of the process (i.e. post-shearing and after one **0.45X** AMPure PB bead purification), is approximately **50%-80%**. | |
| **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 Treatment of DNA

This step improves library quality by removing single-stranded ends from fragments. If preparing larger amounts of DNA, scale the reaction volumes accordingly (i.e., for 10 µg of DNA scale the total volume to 100 µL). Do not exceed 100 ng/µL of DNA in the final reaction.

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

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| Sheared DNA | - | | __ µL for 5.0 µg | - | | |
| 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 | | |
| $H_2O$ | - | | __ µL to adjust to 50.0 µL | - | | |
| Total Volume | | | 50.0 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 37ºC for 15 minutes, then return the reaction to 4ºC.

## Repair DNA Damage

Use the following table to prepare your reaction.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (ExoVII treated) | - | | 50 µL | - | | |
| DNA Damage Repair Mix |  | 25 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 37ºC for 20 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.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (Damage Repaired) | - | | 52.0 µL | - | | |
| End Repair Mix |  | 20 X | 2.5 µL | 1X | | |
| Total Volume | | | 54.5 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, return the reaction to 4ºC.

| STEP | ✓ | Purify DNA | Notes |
|---|---|---|---|
| 1 | | Add **0.45X** volume of AMPure PB beads to the End-Repair reaction. | |
| 2 | | Mix the bead/DNA solution by tapping the tube gently. | |
| 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 gently rotating the tube for 30 minutes at room temperature. Ensure that the bead/sample mixture is mixing throughout the rotation process. | |
| 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. Place in magnetic rack and remove 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 | | Elute the DNA off the beads in **20 µL** Elution Buffer. Mix by tapping the tube gently until the beads are suspended in solution. Rotate the sample gently at room temperature for 20 minutes. | |
| 14 | | Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate. | |
| 15 | | Optional: Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. Note that typical yield at this point of the process (following End-Repair and one **0.45X** AMPure PB bead purification) is approximately between **80-100%** of the total starting material. | |
| 16 | | The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration. | |
| 17 | | Actual recovery per µL and total available sample material: _____ | |

## Prepare Blunt Ligation Reaction

If preparing larger amounts of DNA, scale the reaction volumes accordingly

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.

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|---|
| DNA (End Repaired) | - | | 19.0 µL to 20.0 µL | | | |
| **Annealed** Blunt Adapter (20 µM) |  | 20 µM | 10* 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 | | |
| $H_2O$ | - | - | __ µL to adjust to 40.0 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.

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 overnight.
5. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonuclease after this step.

Add exonuclease 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 | | 1.0 µL |
| ExoVII |  | 10.0 U/µL | | 1.0 µL |
| Total Volume | | | | 42 µL |

1. Spin down contents of tube with a quick spin in a microfuge.
2. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC. You must proceed with purification after this step.

## Purify SMRTbell™ Templates

It is highly recommended to perform two AMPure PB bead purifications after the Exo III and VII digestion.

| STEP | ✓ | First AMPure PB Bead Purification | Notes |
|---|---|---|---|
| **1** | | Add **0.45X** volume of AMPure PB beads to the exonuclease-treated reaction. | |
| **2** | | Mix the bead/DNA solution by tapping the tube gently. | |
| **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 gently rotating for 30 minutes at room temperature. Ensure that the bead/sample mixture is mixing throughout the rotation process. | |
| **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. Place in magnetic rack and | |
| **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** | | Elute the DNA off the beads in **100 µL** Elution Buffer. Mix by tapping the tube gently until the beads are suspended in solution. Rotate the sample gently at room temperature for 20 minutes. | |

| STEP | ✓ | Second AMPure PB Bead Purification | Notes |
|---|---|---|---|
| **1** | | Add **0.45X** volume of AMPure PB beads. | |
| **2** | | Mix the bead/DNA solution by tapping the tube gently. | |
| **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 gently rotating for 30 minutes at room temperature. Ensure that the bead/sample mixture are mixing throughout the rotation process. | |
| **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. Place in magnetic rack and remove 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** | | Elute the DNA off the beads in **20 µL** Elution Buffer. Mix by tapping the tube gently until the beads are suspended in solution. Rotate the sample gently at room temperature for 20 minutes. | |

## BluePippin™ Size-Selection System

Follow the recommendations below and the BluePippin User Manual and Quick Guide (to www.sage-science.com) to size-select the ~20 kb SMRTbell templates using the BluePippin instructions.

| STEP | ✓ | Prepare DNA Samples for Each Lane | Notes |
|---|---|---|---|
| **1** | | If necessary, dilute up to 4 µg SMRTbell template into a final volume of 30 µL Elution Buffer. Run 500 ng to 4 µg SMRTbell templates per lane. It's not recommended to start with less than 500 ng per lane. | |
| **2** | | Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. The Loading Solution is viscous so pipet slowly to ensure it is completely transferred into the DNA sample. Mix thoroughly by gently pipetting; do not vortex. Spin briefly to collect the contents at the bottom. | |
| **3** | | Follow the manufacturer's recommendations for run setup and calibration. | |
| **4** | | Use 0.75%, DF Marker S1 high-pass 15 kb – 20 kb for the run and enter BP start = 15000 and BP end = 50000 bp. | |
| **5** | | Collect 40 µL of the sample in the elution wells into a 1.5 or 2.0 mL LoBind microcentrifuge tube. Wash the well with 40 µL 0.1% Tween20 buffer supplied in the kit. Proceed directly to AMPure PB bead purification.<br>Note: It is highly recommended to wait at least 45 minutes after the run before collecting the eluted DNA. This has shown an increase in recovery of SMRTbell libraries. | |

Concentrate size-selected SMRTbell templates in a 1.5 or 2.0 mL LoBind microcentrifuge tube using two rounds of 0.45X AMPure PB beads purifications.

| STEP | ✓ | AMPure PB Bead Purification | |
|---|---|---|---|
| **1** | | Add **0.45X** volume of AMPure PB beads. | |
| **2** | | Mix the bead/DNA solution by tapping the tube gently. | |
| **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 30 minutes at room temperature. Ensure that the bead/sample mixture are mixing throughout the rotation | |
| **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. Place in magnetic rack and | |
| **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 4 µg input non-size selected library, elute in **23 µl** Elution Buffer. If you size selected more than 4 ug of SMRTbell Template, scale volume of Elution Buffer proportionally (i.e. for up 10 µg of input DNA, elute in **46 µl** EB). Rotate the sample gently at room temperature for 20 minutes. | |
| **14** | | Optional: Verify your DNA amount and concentration using the Qubit quantitation platform, as appropriate. | |
| **15** | | The size selected library can be stored overnight at 4ºC or at -20 ºC for longer duration. | |

## Repair DNA Damage After Size-Selection

Using the table below, set up a reaction to repair any DNA damage present in SMRTBell templates after size-selection. For up to 5 µg of DNA, use a reaction volume of 50 µL. If starting with more than 5 µg of size-selected template, scale reaction volumes proportionally (i.e., for up to 10 µg of DNA use a 100 µL reaction volume).

| Reagent | Tube Cap Color | Stock Conc. | Volume | Final Conc. | | Notes |
|---|---|---|---|---|---|---|
| Size-selected DNA | – | | __ µL for 5.0 µg | – | | |
| 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 |  | 25 X | 2.0 µL | 1X | | |
| $H_2O$ | – | | __ µL to adjust to 50.0 µL | – | | |
| Total Volume | | | 50.0 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 37°C for 60 minutes.

| STEP | ✓ | First AMPure PB Bead Purification | Notes |
|---|---|---|---|
| **1** | | Add **0.45X** volume of AMPure PB beads. | |
| **2** | | Mix the bead/DNA solution by tapping the tube gently. | |
| **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 30 minutes at room temperature. Ensure that the bead/sample mixture are mixing throughout the rotation process. | |
| **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. Place in magnetic rack and remove 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** | | Elute the DNA off the beads in **100 µL** Elution Buffer. Mix by tapping the tube gently until the beads are suspended in solution. Rotate the sample gently at room temperature for 20 minutes. | |

| STEP | ✓ | Second AMPure PB Bead Purification |
|---|---|---|
| 1 | | Add **0.45X** volume of AMPure PB beads. |
| 2 | | Mix the bead/DNA solution by tapping the tube gently. |
| 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 30 minutes at room temperature. Ensure that the bead/sample mixture are mixing throughout the rotation process. |
| 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. Place in magnetic rack and remove 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 | | Elute the DNA off the beads in **10 µL** Elution Buffer. Mix by tapping the tube gently until the beads are suspended in solution. Rotate the sample gently at room temperature for 20 minutes. |
| 14 | | Verify your DNA amount and concentration using Qubit quantitation platform, as appropriate. |

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

Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80ºC. This avoids exposing large insert SMRTbells to heat. The template and primer mix can then be incubated at 20ºC for 30 minutes. Note that you must have the PacBio DNA/Polymerase Kit and use LoBind microcentrifuge tubes for this step.

For polymerase binding, incubation at 30ºC for 4 hours. Instructions for polymerase binding are provided by the calculator.

## Prepare for MagBead Loading

For binding to Magnetic Beads, bind for 30 minutes to 1 hour at 4ºC. Follow the Binding Calculator for instructions on preparing samples for Sequel.

## Sequence

When sequencing size-selected ~20 kb SMRTbell templates prepared by this method, be sure to indicate the magnetic bead collection protocol, a 20,000 bp insert size, collect up to 6 hours with immobilization time of 2 hours.


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