# Procedure & Checklist - 10 kb Template Preparation and Sequencing

## Before You Begin

To perform this procedure, you must have the PacBio® Template Prep Kit.

This procedure can be used to prepare 10 kb libraries from 5 µg of sheared and concentrated DNA. For sheared DNA libraries and PCR products greater than 2 kb, any DNA damage (generated during DNA extraction and PCR amplifications) must be repaired using the DNA Damage Repair reagents provided by Pacific Biosciences and according to the instructions as set forth in this Procedure. Common types of damage may include abasic sites, cytosine deamination, and oxidation.

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).

| Insert Size Target | Insert Size Range | Sheared and Concentrated DNA Amount | Ligation | DNA Damage Repair |
|---|---|---|---|---|
| 10 kb | 8 kb to 12 kb | 5 µg | Blunt | Required |

For DNA sequencing, it is recommended to perform titrations to ensure optimal loading.

## Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear your DNA sample. The most up-to-date guidance on how to use the g- TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device user manual available for download from the Covaris website. 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. 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 ExoVII treatment reaction.

Note that a Hydroshear® Shearing Device can also be used to shear DNA samples. However, because Hydrodynamic shearing performance can vary with each shearing assembly, we recommend optimizing the shearing whenever a new shearing assembly is used.


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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 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.<br>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 shaking 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 vortexing, the bead/DNA mixture should appear homogenous.<br>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.<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. | |

| STEP | ✓ | Concentrate DNA | Notes |
|---|---|---|---|
| **10** | | Remove residual 70% ethanol and dry the bead pellet.<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 the tube caps open) for 30 to 60 seconds. | |
| **13** | | For 5 µg of input sheared gDNA, elute in **36 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 72 µL EB).<br><br>Add the Pacific Biosciences Elution Buffer volume to your beads.<br>– Thoroughly resuspend beads by vortexing for 1 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again.<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 LoBind tube.<br>– Discard the beads.<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. | |
| **14** | | 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><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><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%**. | |
| **15** | | The sheared DNA can be stored for up to 24 hours at 4ºC or at -20ºC for longer duration. | |
| **16** | | 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 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 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. Proceed to the next step.

| STEP | ✓ | Purify DNA | Notes |
|---|---|---|---|
| 1 | | Add **0.45X** 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.<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 **32 µL** Elution Buffer. Mix until homogenous, then vortex for 1 minute at 2000 rpm. If you started with more than 5 µg input sheared DNA, scale volume of EB proportionally (i.e., for 6-10 µg of DNA, elute in 64 µL EB). | |
| 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

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

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. 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) | | | 32.0 µL | | | |
| 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 | | | |

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 15 minutes. At this point, the ligation can be extended up to 24 hours or cooled to 4ºC (for storage of up to 24 hours).
4. 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 | | | | 40.0 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. 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

There are 3 purification steps, 2 using **0.45X** volume of AMPure PB beads and a final purification with either **0.40X** or **0.45X** volumes of AMPure PB beads.

| STEP | ✓ | Purify SMRTbell Templates - First Purification | Notes |
|---|---|---|---|
| **1** | | Add **0.45X** 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.<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 ofthe 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** of Elution Buffer. Vortex for 1 minute at 2000 rpm. | |
| **14** | | The eluted DNA in **100 µL** Elution Buffer should be taken into the second **0.45X** AMPure PB bead purification step. | |

| STEP | ✓ | Purify SMRTbell Templates - Second Purification | Notes |
|---|---|---|---|
| **1** | | Add **45 µL** (**0.45X** volume) of AMPure PB beads to the **100 µL** of eluted DNA from the first AMPure PB bead purification step above. | |
| **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.<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 ofthe 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** of Elution Buffer. Vortex for 1 minute at 2000 rpm. | |
| **14** | | Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. If recovery is sufficient to allow for an additional 25% loss in the final AMPure PB bead purification step (more if the library contains a high number of small fragments), and it is desirable to increase the stringency of size selection, consider using **0.40X** volumes of AMPure PB beads. This will remove most fragments <1.5 kb which will dominate loading, if present. Otherwise, proceed to the third **0.45X** volumes of AMPure PB bead purification step.<br>Note that yield from **0.40X** is typically ~ 10% lower than **0.45X** volumes of AMPure PB bead purification. | |

| STEP | ✓ | Purify SMRTbell Templates - Third Purification | Notes |
|---|---|---|---|
| 1 | | Add **45 µL** (**0.45X** volume) or **40 µL** (**0.40X** volume) of AMPure PB beads to the **100 µL** of eluted DNA. Note that for **0.40X** volume, it is critical to accurately pipet the desired volume of AMPure PB bead solution; there is a steep drop-off in recovery for concentrations <**0.40X**. | |
| 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.<br>Note: It is especially important to save the supernatant for **0.40X** volumes of AMPure PB purification steps. In case of low recovery, perform a **1X** AMPure PB purification step to recover the DNA. | |
| 8 | | Wash beads with freshly prepared 70% ethanol. | |
| 9 | | Repeat step 8 above. | |
| 10 | | Remove residual 70% ethanol and dry the bead pellet.<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** of Elution Buffer. 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). Vortex for 1 minute at 2000 rpm. | |
| 14 | | Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from the Damage Repair input. If your yield concentration is below 12 ng/µL, use the Qubit system for quantitation.<br>To estimate your final concentration:<br>(____ng of DNA going into Damage Repair X 0.2) /__of Elution Buffer =____ng/µL | |
| 15 | | Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process (at the end of library preparation) is between approximately **5-20%** of the total starting DNA amount. | |

## 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 10 kb SMRTbell libraries using P6 polymerase can typically be achieved using an on-plate concentration of 10 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.

| Revision History (Description) | Version | Date |
|---|---|---|
| Added Exo VII pre-treatment to first table on page 4. Specified how much Elution Buffer to use in Concentrate DNA table in step 13 on page 3. Changed some volumes throughout. Updated Annealing and Binding Conditions on last page. | 07 | November 2017 |