Guidelines for Purifying Contaminated SMRTbell™ Libraries Using Magnetic Beads

Purpose

Single molecule, real-time (SMRT®) sequencing is inherently sensitive to the presence of contaminants in DNA samples that may potentially interfere or otherwise completely / incompletely inhibit the sequencing reaction. It is therefore important to have samples of high quality so that sequencing analyses will result in the best possible results. To obtain high-quality samples, purification of DNA prior to making SMRTbell templates is recommended. In the event that a contaminant remains, and sequencing is inhibited, it is desirable to purify the SMRTbell template prior to re-sequencing. This protocol provides a method for cleaning up DNA SMRTbell templates from undesired contaminants using a hybridization/magnetic-bead-based approach.

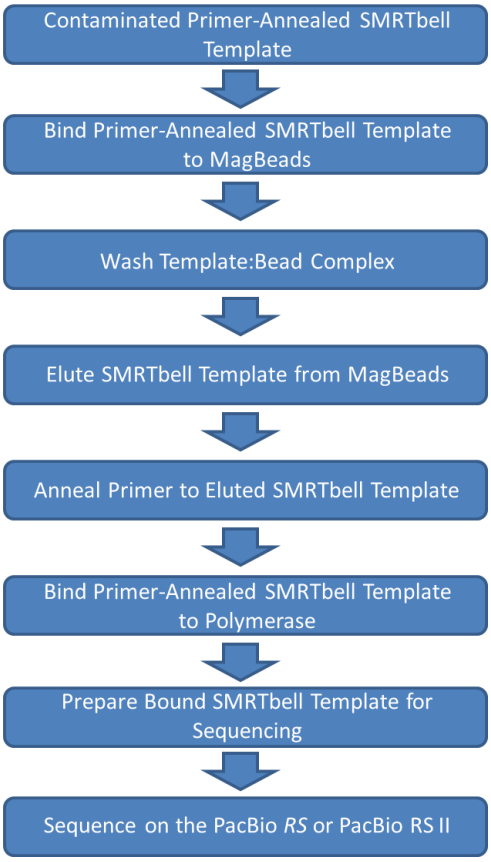
Before You Begin

To perform this procedure you must have the following kits, materials, and instrumentation:

Item	Vendor
DNA Template Prep Kit 2.0	Pacific Biosciences
10X Primer Buffer	Pacific Biosciences (P/N 001-560-849)
Sequencing Primer v2	Pacific Biosciences (P/N 001-560-740)
MagBead Kit	Pacific Biosciences (P/N 100-133-600)
Eppendorf® Thermomixer® or equivalent shaker equipped with temperature control	

Procedural Overview

This SMRTbell library cleanup protocol involves a) binding annealed PacBio DNA SMRTbell libraries to magnetic beads, b) washing the bound annealed DNA SMRTbell templates to remove potential contaminants, and c) eluting the purified, annealed DNA SMRTbell templates from the magnetic beads. The purified SMRTbell templates are then prepared for sequencing on the PacBio *RS* or PacBio RS II instrument.



MagBead Wash

Clean the MagBeads as per PacBio Procedure & Checklist – Low-Input 10 kb Library Preparation and Sequencing (MagBead Station), steps 1-8.

1. Resuspend the beads in the stock bottle by mixing vigorously (30 seconds on a VWR® vortex mixer at high speed). Before aliquoting the MagBeads out of the stock bottle, mix contents by pipetting up and down with a P1000 micropipettor. Aliquot beads into a LoBind® microcentrifuge tube. Each sample requires 100 µl of beads for the cleanup process.

   Note: You can spin down contents of the tube with a quick spin. The beads will pellet at the bottom but are collected on the side of the tube when placed in the magnetic rack.
2. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
3. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.
4. Remove the tube from the magnetic bead rack, and add the MagBead Wash Buffer in the same volume as the bead aliquot.
   a. Vortex the tube for approximately 5 seconds to resuspend beads.
   Quickly spin down the contents of the tube.
5. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
6. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.
7. Remove the tube from the magnetic bead rack, and add the MagBead Binding Buffer in the same volume as the bead aliquot.
   a. Vortex the tube for approximately 5 seconds to resuspend beads.
   b. Quickly spin down the contents of the tube.
8. Place the tube on ice until ready to use.

Annealed SMRTbell Library Cleanup Preparation

9. Enter sample information into the Binding Calculator and make sure to select:
   a. Magnetic Beads: Yes
   b. Preparation Protocol: Small scale
10. Perform annealing as per calculator (0.833 nM template concentration).
11. Place annealed SMRTbell tube on ice until ready to use. Note the volume of annealed SMRTbell template: _____ µl.
12. Dilute annealed SMRTbell template 1:1 by adding equal volume of MagBead Binding Buffer to the tube from **step 11**. Note the volume of diluted SMRTbell template: _____ µl.
    a. Pipette up and down or tap with finger to mix.
    b. Quickly spin down sample.
13. Take the tube containing washed MagBeads (**step 8**) and place in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
14. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.
15. Remove the tube from the magnetic bead rack, and add the diluted annealed SMRTbell template from **step 12**. Mix by pipetting up and down or a quick (5 second) vortex.
16. Place the tube in a rotator and incubate at 4°C for a minimum of 15 minutes (up to 2 hours).

Wash and Recovery of Purified SMRTbell™ Library

17. In a LoBind microcentrifuge tube, make a 1:20 dilution of the 10X Primer Buffer v2 from the DNA Template Prep Kit 2.0. The resulting 0.5X Primer Buffer should be prepared in a sufficient volume for elution (see **step 30**). Set this tube aside at room temperature.
18. Once the beads and annealed SMRTbell template are bound from **step 16**, quickly spin down the tube to collect the beads at the bottom of the tube.

    Notes:

    - It is important to keep sample and reagents cold during the washing process.
    - All washes should use the same volume as the diluted SMRTbell template from **step 12.**

19. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
20. Slowly pipette off cleared supernatant and **save** in an empty LoBind tube. Avoid disturbing the bead pellet.
    a. This tube should be saved until yield is determined. If yield from the process is low, this tube can be used to help troubleshoot loss.
21. Remove the tube from the magnetic bead rack, and add the MagBead Binding Buffer in the appropriate volume (see **Note** above).
    b. Vortex the tube for approximately 5 seconds to resuspend beads.
    c. Quickly spin down the contents of the tube.
22. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
23. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.
24. Remove the tube from the magnetic bead rack, and add the MagBead Wash Buffer in the appropriate volume (see **Note** above).
    d. Vortex the tube for approximately 5 seconds to resuspend beads.
    e. Quickly spin down the contents of the tube.
25. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
26. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.
27. Remove the tube from the magnetic bead rack, and add the MagBead Binding Buffer in the appropriate volume (see **Note** above).
    f. Vortex the tube for approximately 5 seconds to resuspend beads.
    g. Quickly spin down the contents of the tube.
28. Place the tube in a magnetic bead rack until the beads collect to the side of the tube (approximately 30 seconds).
29. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.

30. Remove the tube from the magnetic bead rack, and add the 0.5X Primer Buffer made in **step 17** in a volume approximately ½ of the volume of the initial annealed SMRTbell template volume from **step 11**. It is not recommended to elute in less than 10 µl volume.
    h. Vortex the tube for approximately 5 seconds to resuspend beads.
    i. Quickly spin down the contents of the tube.
31. Incubate at 30°C for 10 minutes in an Eppendorf Thermomixer shaking at 1000 rpm.

    If an Eppendorf Thermomixer is not available, keep sample and beads in a LoBind tube and incubate in a thermal cycler, taking the tube out to quickly vortex every 2 minutes.
32. Once incubation is complete, the tube should be immediately placed on a magnetic bead rack until the beads collect to the side of the tube.

    Note: This step must be performed while the solution is warm.
33. Slowly pipette off cleared supernatant and save in a LoBind tube. **This supernatant contains the purified SMRTbell library**.
34. Measure the concentration of recovered SMRTbell template using Qubit® Fluorometric Quantitation. Typical recovery is >50%.
    j. If recovery is much lower, go back to the saved supernatant from **step 20** and perform quantitation on the supernatant. If most of the DNA is present in the supernatant, begin the process again from **step 1**.
35. Re-enter sample information into the annealing and binding calculator using the new SMRTbell template concentration.
36. Using the new SMRTbell library concentration, perform the annealing, binding and sequencing setup steps as recommended by the calculator. Note that non-standard annealing and binding conditions may need to be employed if the concentration of recovered SMRTbell library (from **step 34**) is significantly lower than expected.