Procedure & Checklist - cDNA Capture Using SeqCap® EZ Libraries

Before You Begin

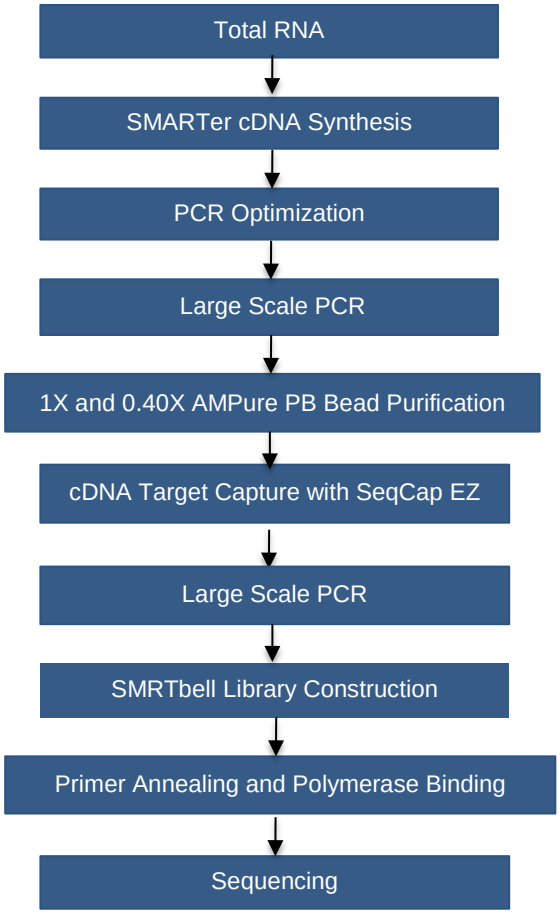
This document describes the process for capturing cDNA prepared with the SMARTer PCR cDNA Synthesis Kit (Clontech) and pulled-down using SeqCap EZ Libraries (Roche NimbleGen) for sequencing on the PacBio® System.

To perform this procedure, you must have reviewed the Procedure & Checklist – Iso-Seq Template Preparation for Sequel Systems.

Workflow

The workflow includes the following:

1. Preparing the cDNA library using the SMARTer PCR cDNA Synthesis Kit.
2. Capturing cDNA with the SeqCap EZ Library (biotinylated probes).
3. Constructing SMRTbell® libraries.
4. Sequencing using the PacBio System.



Part Number 101-601-200 Version 01 (June 2018)

Materials Needed

Item	Vendor	Part Number
cDNA Library		
SMARTer PCR cDNA Synthesis Kit	Clontech	
Lonza flash gel	Lonza	
Target Capture		
PolyT blocker Oligo (5' TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT	IDT	N/A
SeqCap EZ Hybridization and Wash Kit	NimbleGen/Roche	5634261001
Takara LA Taq DNA Polymerase Hot-Start version	Clontech	RR042A
SMARTer PCR Oligo (5' AAG CAG TGG TAT CAA CGC AGA GTA C	IDT	N/A
Dynabeads® M-270 Streptavidin	Life Technologies	65305
SMRTbell Library Construction and Sequencing		
Template Prep Kit	PacBio	
DNA/Polymerase Binding Kit	PacBio	
DNA Sequencing Kit	PacBio	
AMPure® PB beads	PacBio	

Prepare cDNA Library

To prepare a cDNA library, refer to pages 1 - 11 of the Procedure & Checklist – Iso-Seq Template Preparation for Sequel Systems.

1. Prepare First-strand synthesis using the SMARTer PCR cDNA Synthesis Kit
2. Enrich by:
   a. Optimizing PCR cycles.
   b. Performing large-scale PCR.

cDNA Capture using SeqCap EZ Probes

<table>
<tr><th>STEP</th><th>✓</th><th>Prepare the Sample for Hybridization</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>In this section, you will need the following:
SMARTer PCR oligo (IDT)
PolyT blocker (IDT)
2X Hybridization Buffer (tube 5) contained in SeqCap EZ Hybridization and Wash Kit
Hybridization Component A (tube 6) found in SeqCap EZ
Hybridization and Wash Kit
EZ Library (target probes)</td><td></td></tr>
<tr><td>2</td><td></td><td>Add 1.5 µg cDNA to a new 1.5 mL LoBind tube.</td><td></td></tr>
<tr><td>3</td><td></td><td>Add 1 µL of SMARTer PCR oligo and 1 µL PolyT blocker (both at 1000 µM) to the LoBind tube containing the cDNA.</td><td></td></tr>
<tr><td>4</td><td></td><td>Close the tube’s lid and puncture a hole in the cap with an 18 – 20 gauge or smaller needle.</td><td></td></tr>
<tr><td>5</td><td></td><td>Dry the cDNA Sample Library/SMARTer PCR oligo/PolyT blocker completely in a DNA vacuum concentrator (speed vac).</td><td></td></tr>
<tr><td>6</td><td></td><td>To the dried-down sample add:<table><tr><th>Component</th><th>Volume</th></tr><tr><td>2X Hybridization Buffer – tube 5</td><td>7.5 µL</td></tr><tr><td>Hybridization Component A – tube 6</td><td>3 µL</td></tr></table></td><td></td></tr>
<tr><td>7</td><td></td><td>Seal the hole in the tube’s cap with a laboratory tape.</td><td></td></tr>
<tr><td>8</td><td></td><td>Mix the reaction by tapping the tube, followed by a quick spin.</td><td></td></tr>
<tr><td>9</td><td></td><td>Place the tube in a +95℃ h eat block for 10 minutes to denature the cDNA.</td><td></td></tr>
<tr><td>10</td><td></td><td>Quick spin at maximum speed, allowing the mix to cool to room temperature before addition of probes. Probes should never be added while at 95°C.</td><td></td></tr>
<tr><td>11</td><td></td><td>Transfer 10.5 µL of the sample to a 0.2 mL PCR LoBind tube containing a 4.5 µL aliquot of EZ Library (probes).</td><td></td></tr>
<tr><td>12</td><td></td><td>Mix and quick spin.</td><td></td></tr>
<tr><td>13</td><td></td><td>Incubate in a thermocycler at +47°C for 16 - 20 hours. The thermocycler’s heated lid should be turned on and set to maintain +57°C (10°C above the hybridization temperature).</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Preparing Beads for Capture</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>In this section, you will need the following:
Tubes 1, 2, 3, 4 and 7 found in the SeqCap EZ Hybridization and Wash Kit
Dynabeads M-270 Streptavidin</td><td></td></tr>
<tr><td>2</td><td></td><td>Prepare Wash Buffers:
a. Label five 1.5 ml LoBind tubes as Buffer 1, 2, 3, 4, and 7 and prepare the following 1X working concentrations:
<table><tr><th>Buffer</th><th>Stock Conc.</th><th>Vol. Buffer</th><th>Vol. Water</th><th>Total Volume*</th><th>Final Conc.</th></tr><tr><td>Wash Buffer I (tube 1)</td><td>10X</td><td>30 µL</td><td>270 µL</td><td>300 µL</td><td>1X</td></tr><tr><td>Wash Buffer II (tube 2)</td><td>10X</td><td>20 µL</td><td>180 µL</td><td>200 µL</td><td>1X</td></tr><tr><td>Wash Buffer III (tube 3)</td><td>10X</td><td>20 µL</td><td>180 µL</td><td>200 µL</td><td>1X</td></tr><tr><td>Stringent Wash Buffer (tube 4)</td><td>10X</td><td>40 µL</td><td>360 µL</td><td>400 µL</td><td>1X</td></tr><tr><td>Bead Wash Buffer (tube 7)</td><td>2.5X</td><td>200 µL</td><td>300 µL</td><td>500 µL</td><td>1X</td></tr></table>*Store working solutions at room temperature (15ºC to 25ºC) for up to 2 weeks. The volumes in this table are calculated for a single capture; scale up accordingly if multiple hybridization reactions are processed.
b. Preheat the following wash buffers to +47°C in a heat block or water bath:
o 100 µL of 1X Wash Buffer I (tube 1)
o 400 µL of 1X Stringent Wash Buffer (tube 4)</td><td></td></tr>
<tr><td>3</td><td></td><td>Prepare the Capture Beads:
a. Allow the Dynabeads M-270 Streptavidin to warm to room temperature for 30 minutes prior to use.
b. Mix the beads thoroughly by vortexing for 15 seconds.
c. For a single sample, aliquot 50 µL beads for capture into a 1.5 mL LoBind tube. Scale up volume for multiple samples.
d. Place the LoBind tube in a magnetic rack. When the supernatant is clear, remove and discard the supernatant being careful not to disturb the beads. Any remaining traces of liquid will be removed with subsequent wash steps. Note: Allow the Dynabeads to settle for at least 1-2 minutes before removing the supernatant. The Dynabeads are “filmy” and slow to collect to the side of the tube.
e. While the LoBind tube is in the magnetic rack, add 100 µL of 1X Bead Wash Buffer (tube 7). For multiple samples, prepare 100 µL Bead Wash Buffer (tube 7) x number of samples.
f. Remove the tube from the magnetic rack and vortex until the beads are in solution.
g. Quick spin and place the LoBind tube back in the magnetic rack to collect the beads to the side of the tube. Once clear, remove and discard the liquid.
h. Repeat steps e - g for a total of two washes.
i. Resuspend by vortexing the beads in 50 µL of 1X Bead Wash Buffer (tube 7). For multiple samples, scale up accordingly.
j. Place the tube in the magnetic rack to collect beads to the side of the tube. For multiple samples, transfer 50 µL aliquots into new LoBind tubes. Once clear, remove and discard the supernatant.
k. The washed beads are now ready to bind the captured cDNA. Proceed immediately to the next step. Do not allow the capture beads to dry. Small amounts of residual Bead Wash Buffer will not interfere with binding of cDNA to the capture beads.</td><td></td></tr>
</table>

STEP	✓	Binding cDNA to Beads and Wash	Notes
1		Bind DNA to the capture beads: a. To the washed capture beads, transfer the 15 µL probe/sample mixture prepared in the "Preparing hybridization section". b. Mix by tapping the tube until the sample is homogeneous. c. Incubate in a thermomixer set to +47℃ for 45 minutes or transfer the mix to a PCR tube and incubate in a thermocycler (heated lid set to +57°C). Hand mix periodically by gently tapping the tube.	
2		Wash the captured cDNA: a. Pre-heat 1X Wash Buffer (tube 1) and 1X Stringent Wash Buffer (tube 4) to 47℃ . a. After the 45-minute incubation, add 100 µL 1X Wash Buffer I (tube 1), pre-heated to 47℃ , to each capture beads. b. Mix by tapping the tube until the sample is homogeneous. c. If using a PCR tube, transfer the sample to a 1.5 mL LoBind tube. d. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. e. Remove the tube from the magnetic rack and add 200 µL of 1X Stringent Wash Buffer (tube 4) pre-heated to +47℃. Mix by tapping the tube until the sample is homogeneous. Work quickly so that the temperature does not drop below +47℃. f. Incubate at +47°C for 5 minutes. g. Repeat steps d - f for a total of two washes using 1X Stringent Wash Buffer (tube 4) heated to +47℃. h. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. i. Add 200 µL of room temperature 1X Wash Buffer I (tube 1). Hand mix by gently tapping the tube. Quick spin. j. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. k. Add 200 µL of room temperature 1X Wash Buffer II (tube 2) and mix by tapping the tube until sample is homogeneous. Quick Spin. l. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. m. Add 200 µL of room temperature 1X Wash Buffer III (tube 3) and mix by tapping the tube until sample is homogeneous. Quick Spin. n. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. o. Remove the tube from the magnetic rack and add 50 µL of EB. This is enough for two PCR reactions required in the next section. p. Store the beads plus captured samples at -15 to -25℃ or proceed to the next step. It is not necessary to separate the beads from the eluted DNA. The bead/sample mix can be added to the PCR reaction directly.	

<table>
<tr><th>STEP</th><th>✓</th><th>Amplification of Captured cDNA</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>In this section, you will need the following:
Takara LA Taq DNA Polymerase Hot-Start Version from Clontech
SMARTer PCR Oligos (from the Clontech Kit)</td><td></td></tr>
<tr><td>2</td><td></td><td>PacBio recommends using Takara LA Taq DNA Polymerase Hot-Start version for amplification.
a. Assemble the following PCR reaction for a total of 200 µL.
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>Water</td><td>104.5 µL</td></tr>
<tr><td>10x LA PCR Buffer</td><td>20 µL</td></tr>
<tr><td>2.5 mM each dNTPs</td><td>16 µL</td></tr>
<tr><td>SMARTer PCR Oligos (12 µM each)</td><td>8.3 µL</td></tr>
<tr><td>Takara LA Taq DNA polymerase</td><td>1.2 µL</td></tr>
<tr><td>Captured Library</td><td>50 µL</td></tr>
<tr><td>Total</td><td>200 µL</td></tr>
</table>
b. Split the PCR mix into two tubes, 100 µL. It is best to perform the PCR reaction in 100 µL volumes.
c. Amplify using the following PCR protocol:
<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td>1</td><td>95℃</td><td>2 minutes</td></tr>
<tr><td>2</td><td>95℃</td><td>20 seconds</td></tr>
<tr><td>3</td><td>68℃</td><td>10 minutes</td></tr>
<tr><td>4</td><td colspan="2">Repeat steps 2-3, 7 to10 times for a total of 8 to 11 cycles</td></tr>
<tr><td>5</td><td>72℃</td><td>10 minutes</td></tr>
<tr><td>6</td><td>4℃</td><td>Hold</td></tr>
</table></td><td></td></tr>
<tr><td>3</td><td></td><td>After amplification, pool the 100 µL reactions and proceed to the next step to purify products using AMPure PB beads.</td><td></td></tr>
</table>

STEP	✓	Post Amplification Clean Up	Notes
1		Add **1X** AMPure PB beads to the pooled PCR product.	
2		Mix thoroughly by tapping the tube until the sample is homogeneous.	
3		Incubate at room temperature for 10 minutes	
4		Place on magnetic rack until solution clears. Remove and discard supernatant.	
5		With the tube still on magnet, add 200 µL freshly prepared 70% ethanol to the tube.	
6		Remove and discard 70% ethanol.	
7		Repeat steps 5 to 6 for total of two washes with 70% ethanol.	
8		Let beads air dry for 1 minute. (Note - over drying the beads will result in reduced DNA yield.)	
9		Add 27 µL EB and remove the tube from the magnet. Mix by tapping the tube until the sample is homogeneous. Then incubate at room temperature for 2 minutes.	
10		Place back on magnet. When the solution clears, transfer supernatant into new 1.5 mL LoBind tube.	
11		Determine concentration using Qubit device or similar quantification assay.	
12		Run 1 µL of sample on Agilent DNA 12000 chip according to manufacturer's instructions.	
13		The captured cDNA is now ready for SMRTbell library construction.	

Repair DNA Damage

Use the following table to repair any DNA damage. 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 microcentrifµge tube, add the following reagents:

Reagent	Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
cDNA			__ 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			2.0 µL			
H_2O			__ µL to adjust to 50.0* 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 gentle mixing.
3. Spin down contents of LoBind 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
cDNA (Damage Repaired)			50 µL			
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL			

1. Mix the reaction well by gentle mixing.
2. Spin down contents of LoBind 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 **1X** volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution by tapping the tube.	
3		Allow the DNA to bind by letting it sit at room temperature for 10 minutes.	
4		Spin down the LoBind tube (for 1 second) to collect beads.	
5		Place the LoBind tube in a magnetic bead rack to collect the beads to the side of the tube.	
6		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
7		Wash beads with freshly prepared 70% ethanol.	
8		Repeat step 8 above.	
9		Remove residual 70% ethanol and dry the bead pellet. – Remove the LoBind tube from the 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 LoBind tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
11		Remove the LoBind tube from the magnetic bead rack and allow beads to air-dry (with LoBind tube caps open) for 30 to 60 seconds.	
12		Elute the DNA off the beads in **30 µL** Elution Buffer. Mix by gently tapping the LoBind tube until homogenous, then let stand at room temperature for 2 minutes.	
13		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate.	
14		Optional: Perform qualitative and quantitative analysis using a Bioanalyzer system instrument with the DNA 12000 Kit. Note that typical yield at this point of the process (following End-Repair and one **1X** AMPure PB bead purification) is approximately between **80-100%** of the total starting material.	
15		The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	

Prepare Blunt-Ligation Reaction

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

1. In a LoBind microcentrifuge LoBind tube (on ice), add the following reagents in the order shown. Note that you can add water to achieve the desired DNA volume. 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)			29.0 µL to 30.0 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		
H_2O			__ µL to adjust to 40.0 µL			
Total Volume			40.0 µL			

2. Mix the reaction well by gentle mixing.
3. Spin down contents of LoBind tube with a quick spin in a microfuge.
4. 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).
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.

Exo III and Exo VII Treatment

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 LoBind 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

STEP		Purify SMRTbell Templates	Notes
1		Add **1X** volume of AMPure PB beads to the exonuclease-treated reaction.	
2		Mix the bead/DNA solution by tapping the tube.	
4		Allow the DNA to bind to beads by letting the sample sit at room temperature for 10 minutes.	
4		Spin down the LoBind tube (for 1 second) to collect beads.	
5		Place the LoBind tube in a magnetic bead rack to collect the beads to the side of the tube.	
6		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
7		Wash beads with freshly prepared 70% ethanol.	
8		Repeat step 8 above.	
9		Remove residual 70% ethanol and dry the bead pellet. – Remove the LoBind tube from the 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 LoBind tube back on the magnetic bead rack. – Pipette off any remaining 70% ethanol.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
11		Remove the LoBind tube from the magnetic bead rack and allow beads to air-dry (with LoBind tube caps open) for 60 seconds.	
12		Elute the DNA off the beads in **50 µL** of Elution Buffer. Mix thoroughly by gently tapping the LoBind tube and let sit at room temperature for 2 minutes.	
13		The eluted DNA in **50 µL** Elution Buffer should be taken into the second **1X** AMPure PB bead purification step.	

STEP		Second Purification	Notes
1		Add **1X** volume of AMPure PB beads to the **50 µL** of eluted DNA.	
2		Mix the bead/DNA solution by tapping the tube.	
4		Allow the DNA to bind to beads by letting the sample sit at room temperature for 10 minutes.	
4		Spin down the LoBind tube (for 1 second) to collect beads.	
5		Place the LoBind tube in a magnetic bead rack to collect the beads to the side of the tube.	
6		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
7		Wash beads with freshly prepared 70% ethanol.	
8		Repeat step 8 above.	
9		Remove residual 70% ethanol and dry the bead pellet. – Remove the LoBind tube from the 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 LoBind tube back on the magnetic bead rack. – Pipette off any remaining 70% ethanol.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
11		Remove the LoBind tube from the magnetic bead rack and allow beads to air-dry (with LoBind tube caps open) for 60 seconds.	
12		Elute the DNA off the beads in **50 µL** of Elution Buffer. Mix thoroughly by gently tapping the LoBind tube and let sit at room temperature for 2 minutes.	
13		Elute the off the beads in **10 µL**. Mix thoroughly.	
14		Determine concentration using a Qubit device or similar quantification assay.	
15		Run 1 µL of sample on Agilent DNA 12000 chip according to manufacturer's instructions. Perform qualitative analysis using a Bioanalyzer instrument with the DNA 12000 kit. Refer to Agilent Technologies' guides for specific information.	

DNA Control Complex Dilution

You must have the PacBio Control Complex for this step. Dilute the Control Complex according to the volumes and instructions specified in Sample Setup.

Anneal and Bind SMRTbell Templates

Follow the instructions in Sample Setup to anneal and bind your library.

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Revision History (Description)	Version	Date
Initial release. Converted from “Unsupported Protocol” with updates to the cDNA preparation section.	01	June 2018