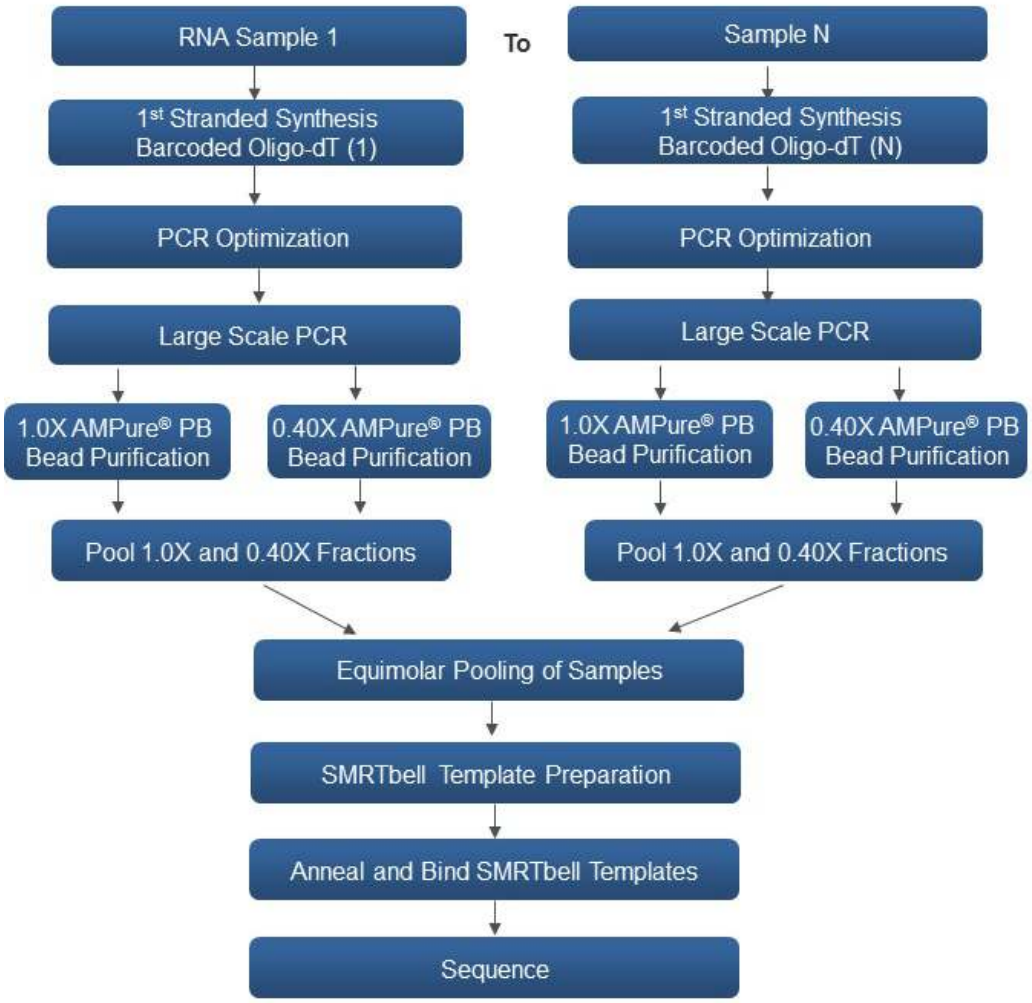
Procedure & Checklist - Multiplex Isoform Sequencing (Iso-Seq Analysis)

Before You Begin

Review the template preparation procedure for Iso-Seq® analysis here. The procedure provides instructions for constructing both a non-size selected library and a size-selected library using BluePippin or SageELF for sequencing in the Sequel® System.

In this document, a workflow for multiplexing RNA samples for sequencing in the Sequel System is provided. RNA samples are barcoded during 1st-strand synthesis using barcoded oligo-dT. A set of barcoded oligo dTs recommended for 1st-strand synthesis are listed in Table 1. After synthesis, each barcoded cDNA is enriched by PCR and subsequently pooled for SMRTbell® library construction.

PacBio recommends starting with 4 - 8 samples for multiplexing. As you get comfortable with the workflow, you can increase the number of samples and use up to 24 barcodes.



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Materials and Kits Needed

Item	Vendor
SMARTer PCR cDNA Synthesis Kit	Clontech (634925 or 634926)
PrimeSTAR GXL DNA Polymerase	Clontech (R050A or R050B)
Barcoded Oligo-dT* (see “Recommended Barcoded Oligo-dT” section on the next page)	Any oligo Synthesis Vendor
Additional 5’ PCR Primer IIA 5’ AAG CAG TGG TAT CAA CGC AGA GTA C 3’	Any Oligo Synthesis Vendor
1.2% FlashGel® system or 0.80% Agarose Gels	Lonza (57023) or (57029) or Any MLS
FlashGel DNA Marker (100 bp – 3 kb or 100 bp - 4 kb)	Lonza
Qubit® dsDNA BR Assay Kit or HS Assay Kit	Invitrogen
DNA 12000 Kit or HS DNA Kit	Agilent
Template Prep Kit DNA/Polymerase Binding Kit DNA Sequencing Kit AMPure® PB Beads	Pacific Biosciences
Optional for Size Selection: ● Blue Pippin System and Consumables: o BluePippin system with Software v5.90 or later o PacBio SMRTbell cassette definition set “0.75% DF 2 –6 kb Marker S1” o 0.75% Dye-Free Agarose Gel Cassettes Loading Solution o S1 Marker o Electrophoresis Buffer ● SageELF System and Consumables: o SageELF system with Software v0.57 or later o PacBio SMRTbell cassette definition set “0.75% Agarose, 1 kb-18 kb” o 0.75% Dye-Free Agarose Gel Cassettes o Loading Solution o DNA Marker	Sage Science

*Recommended Barcoded Oligo-dT

Use any of the recommended barcoded oligo-dT's listed in the table below. The structure of the oligo is as follows:

Primer Sequence 16-bp barcode oligo dT

5'AAGCAGTGGTATCAACGCAGAGTACtcagacgatgcgtcatTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN3'

Name	Sequence	Scale	Purification
dT_BC1001_PB	AAGCAGTGGTATCAACGCAGAGTACCACATATCAGAGTGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1002_PB	AAGCAGTGGTATCAACGCAGAGTACACACACAGACTGTGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1003_PB	AAGCAGTGGTATCAACGCAGAGTACACACATCTCGTGAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1004_PB	AAGCAGTGGTATCAACGCAGAGTACCACGCACACACGCGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1005_PB	AAGCAGTGGTATCAACGCAGAGTACCACTCGACTCTCGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1006_PB	AAGCAGTGGTATCAACGCAGAGTACCATATATATCAGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1007_PB	AAGCAGTGGTATCAACGCAGAGTACTCTGTATCTCTATGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1008_PB	AAGCAGTGGTATCAACGCAGAGTACACAGTCGAGCGCTGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1009_PB	AAGCAGTGGTATCAACGCAGAGTACACACACGCGAGACAGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1010_PB	AAGCAGTGGTATCAACGCAGAGTACACGCGCTATCTCAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1011_PB	AAGCAGTGGTATCAACGCAGAGTACCTATACGTATATCTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1012_PB	AAGCAGTGGTATCAACGCAGAGTACACACTAGATCGCGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1013_PB	AAGCAGTGGTATCAACGCAGAGTACCTCTCGCATACGCGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1014_PB	AAGCAGTGGTATCAACGCAGAGTACCTCACTACGCGCGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1015_PB	AAGCAGTGGTATCAACGCAGAGTACCGCATGACACGTGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1016_PB	AAGCAGTGGTATCAACGCAGAGTACCATAGAGAGATAGTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1017_PB	AAGCAGTGGTATCAACGCAGAGTACCACACGCGCGCTATATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1018_PB	AAGCAGTGGTATCAACGCAGAGTACTCACGTGCTCACTGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1019_PB	AAGCAGTGGTATCAACGCAGAGTACACACACTCTATCAGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1020_PB	AAGCAGTGGTATCAACGCAGAGTACCACGACACGACGATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1021_PB	AAGCAGTGGTATCAACGCAGAGTACCTATACATAGTGATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1022_PB	AAGCAGTGGTATCAACGCAGAGTACCACTCACGTGTGATATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1023_PB	AAGCAGTGGTATCAACGCAGAGTACCAGAGAGATATCTCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_BC1024_PB	AAGCAGTGGTATCAACGCAGAGTACCATGTAGAGCAGAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE

Table 1. Recommended Barcoded Oligo-dT for Iso-Seq Multiplexing

First Strand cDNA Synthesis

For each RNA sample, perform 1st-strand synthesis described on pages 4-5 in the *Procedure & Checklist - Iso-Seq Template Preparation for Sequel System*.

1. Prepare a 12 µM working solution of the barcoded oligo-dTs.
2. For each RNA sample, use the barcoded oligo-dT instead of the 3' SMART CDS primer IIA supplied in the SMARTer PCR cDNA Synthesis Kit.
3. When preparing 1st-strand synthesis reactions, do not change the size (volumes) and conditions of any of the reactions. If necessary, prepare more single tube reactions instead of scaling the reaction volumes.

PCR Optimization and Large-Scale PCR

Each barcoded sample is then enriched by PCR (see pages 5 - 7 in the *Procedure & Checklist - Iso-Seq Template Preparation for Sequel System*).

1. Optimize the number of cycles to generate a good cDNA library (see pages 5-6).
2. Perform large-scale PCR for each sample (page 7)
3. Proceed to AMPure Purification.

1X and 0.40X AMPure Purification

1. For each sample, two fractions are purified using 1X and 0.40X AMPure PB beads (see pages 8 – 11).
2. For each sample, pool 1X and 0.40X (equimolar) fractions (see page 11).
3. Measure the concentration using a Qubit System and determine size of each barcoded sample by running 1 µL in the Bioanalyzer System.
4. Proceed to the next section to pool samples for SMRTbell library construction.

Sample Pooling

Barcoded cDNAs are now ready for equimolar pooling.

STEP		cDNA Sample Pooling	Notes
1		Use the concentration and distribution from the previous step to determine the molarity of each sample. Use the following equation to determine Molarity: $\frac{\text{concentration in ng/ul X } 10^6}{\text{(660 g/mol x average library size in bp*)}}$ = concentration in nM *To determine the average library size, select the region of interest by defining the start and end points of the smear.	
2		Pool equal molar quantities of the barcoded cDNA in a clean LoBind microcentrifuge tube. The total combined mass must not exceed 5 µg.	
3		The pooled cDNA can now be constructed to a SMRTbell library as a single sample. Proceed to SMRTbell Template Preparation described on pages 12 - 17 in the *Procedure & Checklist - Iso-Seq Template Preparation for Sequel System*.	

Data Analysis

For data analysis, refer to the best practices captured **here**.

Revision History (Description)	Version	Date
Initial release. Converted from “Unsupported Protocol” with updates to the Iso-Seq analysis workflow for multiplexing for the Sequel System.	01	June 2018

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