I. Estimating no. of SMRT Cells for Iso-Seq Application (Table 1)

The table below provides general guidelines for the number of SMRT Cells needed for experimental design goals. Number of SMRT Cells for each project is defined per size-selected library and depends on the research goals and transcriptome complexity. For insert sizes >3 kb, the estimated number of SMRT Cells recommended is based on implementing the double BluePippin size-selection protocol, which improves sequencing performance for these longer transcript lengths. The number of SMRT Cells required generally increases for libraries that are not cleanly size-selected (due to the presence of shorter transcripts that were not removed from each size bin). Approximately 25,000 full-length transcripts can be sequenced with each SMRT Cell under optimized sample loading conditions.

Number of SMRT Cells (per sample)	Experimental Goals
1 SMRT Cell	Targeted, gene-specific isoform characterization. (e.g., using PCR products as input). Multiple genes may be analyzed on a single SMRT Cell by employing an appropriate PacBio Barcoding strategy.
1-4 SMRT Cells	General survey of full-length isoforms in a transcriptome using a no-size-selection protocol. Resulting transcript sizes may be skewed towards representation in favor of shorter isoforms (i.e., 1-3 kb).
4-8 SMRT Cells	A focused study on one or two size fractions of full-length transcripts with 4 SMRT Cells per fraction (e.g. 2-3 kb and 3-6 kb).
16 SMRT Cells	A more comprehensive survey of full-length isoforms in the whole transcriptome ranging from 1 kb to 10 kb. General guidance is to run 4 SMRT Cells per size-selected library: e.g., (a) 1-2 kb; (b) 2-3 kb; (c) 3-6 kb; and (d) 6-10 kb library size bins are recommended for enabling coverage of highly expressed isoforms.
>16 SMRT Cells	Add additional sequencing of specific size-selected libraries post-analysis to saturate isoform discovery, or increase the dynamic range for very low abundance transcript discovery and detection.

II. RNA sample requirements for PacBio sequencing

The Iso-Seq Application from Pacific Biosciences provides researchers the ability to sequence intact, full-length transcripts with the demonstrated detection of 5'/3' ends and a polyA+ tail. Project success is highly dependent on the quality of the starting RNA / cDNA material since any damages to transcripts will be directly reflected in the sequencing results. High-quality RNA extractions and clean size-selection of samples are imperative for obtaining long read lengths and optimal sequencing performance.

III. Important measures impacting RNA input sample quality

For optimal sequencing performance, it is **essential** that the RNA sample:

- Has not undergone multiple freeze-thaw cycles as they can lead to additional RNA damage.
- Has not been exposed to high temperatures (*e.g.*: > 65°C for 1 hour can cause a detectable decrease in sequence quality) or pH extremes (< 6 or > 9).
- Has an OD_{260}/OD_{280} ratio between 2.0 and 2.2.

- Has an OD_{260}/OD_{230} ratio between 1.8 and 2.1.
- Has a RIN number ≥ 9 (Recommended).
- Does not contain insoluble material.
- Does not contain DNA contamination.
- Has not been exposed to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes are not RNA damaging, but do avoid ethidium bromide.
- Does not contain denaturants (e.g., guanidinium salts or phenol) or detergents (e.g., SDS or Triton-X100).
- Does not contain carryover contamination from the original organism/tissue (e.g., heme, humic acid, polyphenols, etc.).
- Note: RNA samples should only be shipped with dry ice.

IV. Important measures impacting cDNA input sample quality

The most important factor impacting the quality of cDNA libraries is the quality of the starting RNA (see Section III above).

Please take all precautions against additional damages and ensure that the cDNA sample:

- Has not been exposed to the same types of damaging agents, conditions, and contaminants listed above for RNA input samples (see Section III above).
- Has an OD_{260}/OD_{280} ratio of 1.8 to 2.0.

V. Recommendations for RNA extraction/purification (not an official endorsement from PacBio)

The minimum starting amount is 2 ng of total RNA or 1 ng of polyA+ RNA. The following kits are recommended for polyA+ extraction/purification:

- Ambion® Poly(A) Purist™ MAG Kit (http://products.invitrogen.com/ivgn/product/AM1922)
- Qiagen® RNeasy Plus Kits (http://www.qiagen.com/qdm/rna/rneasy-plus-kits?cmpid=Qven10GARneasy)

VI. Recommendations for cDNA library construction

- Clontech® SMARTer® PCR cDNA Synthesis Kits (http://www.clontech.com/US/Products/cDNA_Synthesis_and_Library_Construction/cDNA_Synthesis_Kits/SMARTer_Kits)
- PacBio User Bulletin – Guidelines for Preparing cDNA Libraries for Isoform Sequencing (Iso-Seq™)

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