Sample preparation instructions for use on the PacBio RSII / Sequel instruments

Single molecule sequencing on the PacBio® instruments requires stringent sample preparation procedures. Since amplification is not required, it is critical to maintain the highest sample quality in order to maximize both sequencing success and its output.

>All new projects must be discussed with us before sending the samples.<

General requirements for Sample Submission

- Sample storage at maximum -20°C
- Avoid freeze-thaw cycles
- Do not expose samples to temperatures above 65°C / pH extremes (<6 or >9)
- OD260/280 between 1.8 and 2.0
- OD260/230 between 2 – 2.2
- There must not be RNA contamination
- Do not expose samples to UV or intercalating fluorsescent dyes
- Ensure that there are no chelating agents (e.g. EDTA), divalent metal ions (e.g. Mg2+), denaturants, or detergents left in the sample (note: EDTA concentration in 0.1x TE buffer is OK)

Always submit the following information

- Gel picture (0.8 % agarose for genomic, 1-1.5 % for amplicons; always add a size marker) or BioAnalyzer result to visualize DNA quality
- OD260/280 and OD260/230 ratios
- Estimation of concentration, name the measurement instrument*
- Information about the extraction protocol
- Type of solvent that DNA is stored in (preferably, 0.1x TE or EB buffer)
- Use low bind microcentrifuge tubes
- Filled in submission forms (obtained from our homepage): as a print out together with the sample, as well as an electronic version to quedenau@mdc-berlin.de

*If using a UV absorbance quantitation method (i.e. Nanodrop or OD260) please submit more material as these methods tend to read up to 2x higher than fluorometric quantitation methods.

Specific applications

DNA-seq: quantity and quality; sample-specific issues

We will require a variable amount of good quality DNA depending on the size of your insert for sequencing. Please consult the table below for estimates of the material needed. Please be aware that excess DNA is needed since initial fragmentation and AmPure bead concentration/clean up steps can be subject to unavoidable template loss.

Library Insert Size	Amount of DNA Required**
Amplicon 250-750bp	250-500ng
Amplicon 750bp-10kb	1ug
3-20 Kb	3-5ug
Size Selected (10-35kb)	5-8ug

**If the template is very limited/low, we can process these samples using our low-input protocols. The latter are not without their disadvantages and are only recommended where absolutely needed.

Any remaining DNA left may be returned to the user if needed. Often the loss of template is less than expected and so if unavoidable, lower input amounts can be accommodated.

Upon library preparation we can then provide you with an appropriate answer as to how many SMRT cells can be loaded using your specific library.

For de novo projects with PacBio data alone, 75-100x sequencing is recommended. This is easily achieved for genomes no larger than 500 Mbp. Larger genomes may also be accommodated, however specific plans must be made with the project coordinator.
For re-sequencing and de novo sequencing in combination with short-read data (Illumina or Ion), 10-20x sequencing depth is recommended depending on the genome size.

RNA-seq (Iso-seq)

Iso-seq allows sequencing of full-length transcripts from the 5' end to the 3'end. This technique is proving to be invaluable for the annotation of de novo genomes. Furthermore, iso-seq is unequivocally contributing to the discovery and analysis of various isoforms both novel and rare.

The user must prepare their own cDNA containing full-length transcripts using the SMARTer PCR cDNA synthesis kit from Clontech. Input RNA must be of a high quality and rRNA should be removed from the sample by either polyA-selection with a kit of choice, or by rRNA depletion of total RNA with an organism-appropriate RiboZero or RiboMinus kit (RiboZero is usually more efficient for bacterial and fungal samples). The Clontech kit can accommodate very low input RNA amounts and so is suitable for applications where RNA is limited. A minimum 2ng total RNA or 1ng polyA RNA can be used. If material is not limited, we do recommend using 1ug total RNA or 0.5ug polyA RNA for cDNA synthesis (please see kit for details). For subsequent amplification steps, it is of upmost importance not to over amplify your template. (Please follow the recommendations as outlined the PacBio isoseq protocol at their website.)

The amount of SMRT cells to be run or coverage needed depends on your scientific question.

As a guide:

RS II SMRT Cells (per sample)	**Sequel** SMRT Cells (per sample)	Experimental Goals
1	<1	Targeted, gene-specific isoform characterization
1-8	1	General survey of full-length isoforms in a transcriptome(moderate to high expression levels)with or without size selection
12-16	1-2	A comprehensive survey of full-length isoforms in the transcriptome across 3-4 size fractions
>16	>2	Deep sequencing for comprehensive isoform discovery and identification of low abundance transcripts across 3-4 size fractions

Of course this is very sample dependent and specific plans can be made to run a pilot project of your samples before running extra SMRT cells.

For barcoding in IsoSeq projects contact us please *before* you start preparing cDNA.

Amplicon-seq

Sequencing of amplicons does not require as much input DNA since no fragmentation is need prior to library preparation.
Pacbio sequencers can process amplicons from 250 bp in size and upward. Barcoding and pooling of amplicons is also possible; please contact us for more details prior to designing your experiment. At least 200-500 ng of short amplicons (up to 1 kb) or 1 ug of long amplicons should be submitted. If concentration is an issue, smaller inputs can be accommodated through the use of low input protocols. These protocols do have their disadvantages must be discussed prior to sequencing.