A Low DNA Input Protocol for High-quality PacBio *de novo* Genome Assemblies

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Abstract

A high-quality reference genome is an essential tool for studying the genetics of traits and disease, organismal, comparative and conservation biology, and population genomics. PacBio Single Molecule, Real-Time (SMRT) Sequencing generates long reads with uniform coverage and high consensus accuracy, making it a powerful technology for *de novo* genome assembly. Improvements in throughput and concomitant reductions in cost have made PacBio an attractive core technology for many large genome initiatives. However, relatively high DNA input requirements (3 µg for standard library protocol) have placed PacBio out of reach for many projects on small organisms that may have lower DNA content or on projects with limited input DNA for other reasons.

Here we present a modified SMRTbell library construction protocol without DNA shearing or size selection that can be used to generate a SMRTbell library from just 150 ng of starting genomic DNA. Remarkably, the protocol enables high quality *de novo* assemblies from single invertebrate individuals and is applied to taxonomically diverse samples. By sequencing and assembling material from a single diploid individual, only two haplotypes are present, simplifying the assembly process compared to samples from multiple pooled individuals.

The libraries were run on the Sequel System with chemistry v3.0 and software v6.0, generating ~11 Gb of sequence per SMRT Cell with 10 hour movies, and followed by *de novo* genome assembly with FALCON. The resulting assemblies had high contiguity (contig N50s over 1 Mb) and completeness (as determined by conserved BUSCO gene analysis) when at least 30-fold unique molecular coverage is obtained.

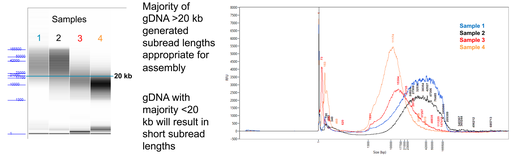
This new low-input approach now puts PacBio-based assemblies in reach for small highly heterozygous organisms that comprise much of the diversity of life. The method presented here is scalable and can be applied to samples with starting DNA amounts of 150 ng per 300 Mb genome size.

SMRTbell Library Preparation Workflow

- Genomic DNA (150 ng)
- Remove SS Overhangs — 37°C for 15 min
- DNA Damage Repair — 37°C for 30 min
- End-Repair/A-tail — 20°C for 10 min, 65°C for 30 min
- Ligation — 20°C for 60 min
- 2 AMPure Purifications — 45 mins
- Primer Annealing, Polymerase Binding Sequencing

- DNA extraction with MagAttract HMW DNA Kit yields partially fragmented DNA suitable for library construction.
- SMRTbell Express Template Prep 2.0 is a single-tube, addition-only workflow eliminating buffer exchanges, minimizing DNA loss.
- No DNA shearing or size selection.
- 2 rounds 0.45X Ampure Purification
- Yield >50%, >4 SMRT Cells
- ~3.5 hours preparation time

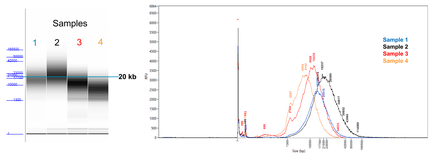
DNA Requirements



Majority of gDNA >20 kb generated subread lengths appropriate for assembly

gDNA with majority <20 kb will result in short subread lengths

Figure 1. Quality of DNA for library construction. FEMTO *Pulse* gel images and traces of 4 gDNA samples. DNA 1 and 2 contain fragments with majority of DNA >20 kb (minimal fragments <20 kb) suitable for generating long reads for *de novo* assembly. DNA 3 and 4 are too fragmented with majority of DNA <20 kb and not recommended for library construction and sequencing.



Sample	Library Size (kb)	Mean Subread Length (kb)	N50 Subread Length (kb)
1	17	8.2	12
2	22	14.5	13
3	12	4.3	7
4	9	3.8	6

Figure 2. Impact of <10 kb short insert SMRTbells to subread length. FEMTO *Pulse* gel images and traces of 4 SMRTbell libraries. The majority of inserts for libraries 3 and 4 are <10 kb, resulting in ~4 kb subread lengths.

Example: Parasitic Flatworm *(Schistosoma mansoni)*

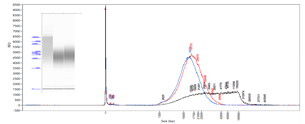


Figure 3. *Schistosoma mansoni* input DNA and two resulting libraries. Femto *Pulse* QC traces and gel images (inset) of the genomic DNA input (black) and two final libraries (blue: 100 ng input, yield 50%; red: 45 ng input, yield 55%). The second library (red) was used for sequencing.

Loading Conc.	Total Yield (Gb)	Unique Mol. Yield (Gb)	N50 Subread Length	P0	P1	P2
4 pM	32.3	3.8	13,437	47.3%	44.3%	8.5%
4 pM	26.9	3.2	13,351	54.3	36.3%	9.4%
4 pM	32.5	3.8	13,586	50.7%	39.9%	9.4%
4 pM*	20.6	3.8	12,121	34.1%	52.7%	13.2%

Table 1. Run statistics for the four Sequel SMRT Cells run from the library. (Chemistry 3.0, SW 6.0, 20 hour runs/Cell; *10 hour run for the last Cell). A total of 41 X UMC was generated for assembly.

Table 2. Assembly statistics of the PacBio *Schistosoma mansoni de novo* assembly, and compared with the previous assembly for this species (GCA_000237925.2). BUSCO was run on the combined primary contig and haplotigs PacBio assembly.

		PacBio	2012 reference
Contigs	Total Length	382 Mb	364 Mb
	No. contigs	327	9,516
	Contig N50	3.8 Mb	0.077 Mb
BUSCO (eukaryota, N=303)	Complete	86.1 %	84.8 %
	Duplicate	17.8 %	3.0 %
	Fragmented	4.0 %	5.3 %
	Missing	9.9 %	9.9 %

Example: Malaria Mosquito (*Anopheles coluzzii)*

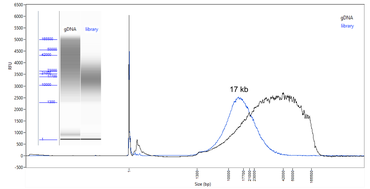


Figure 4. *Anopheles coluzzii* input DNA and final SMRTbell library. Majority of gDNA is >20 kb and the final SMRTbell library is 17 kb. 100 ng input gDNA.

Loading Conc.	Total Yield (Gb)	Unique Mol. Yield (Gb)	N50 Subread Length	P0	P1	P2
5 pM	24.1	4.5	12,978	26.0%	60.1%	13.9%
5 pM	23.6	4.5	13,132	27.1%	59.0%	14.0%
6 pM	25.0	3.9	12,751	35.3%	53.1%	11.7%

Table 3. Run statistics for the three Sequel SMRT Cells run from the library (Chemistry 3.0, SW 6.0, 20 hour movie/SMRT Cell). A total of 45-fold unique molecular coverage (UMC) was generated for assembly.

		PacBio	Sanger assembly
Contigs	Total Length	251 Mb	224 Mb
	No. contigs	206	27,063
	Contig N50	3.47 Mb	0.025 Mb
BUSCO (diptera N=2799)	Complete	98.1 %	87.5 %
	Duplicate	2.4 %	0.1 %
	Fragmented	0.9 %	6.8 %
	Missing	1.0 %	5.7 %

Table 4. Assembly statistics of the PacBio *Anopheles coluzzii de novo* assembly, and compared with the previous Sanger-sequence based assembly for this species from [1] (GCA_000150765.1). BUSCO [2] was run on the PacBio primary contigs after curation with Purge Haplotigs [3].

Conclusion

- Single-tube, addition-only workflow with SMRTbell Express Template Prep 2.0 minimizes DNA loss, enabling library construction and high-quality *de novo* genome assemblies from as little as 150 ng input gDNA.
- Size-selection is not required if majority of gDNA is >20 kb.
- While the data presented here were generated from 20-hour movies, 10-hour movies with 120 min pre-extension is appropriate.
- Enables sequencing single individuals with no need for inbreeding or pooling multiple samples.
- For more detail, see Kingan et al. (2019). A High-Quality *De novo* Genome Assembly from a Single Mosquito Using PacBio Sequencing. *Genes*, *10*(1), 62 https://doi.org/10.3390/genes10010062

References

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2. Simão FA et al. (2015) BUSCO: assessing genome assembly and annotation completeness with single- copy orthologs. *Bioinformatics*. 31(19), 3210-3212.
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4. https://www.vectorbase.org/organisms/anopheles-gambiae/pest/agamp4 [cited 7 Aug 2018]