Getting a Read on One Little Worm with PacBio’s Low DNA Input Workflow and the Agilent Femto Pulse System

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University of Delaware

Product Note
Workflow Solutions

Fast, High-Resolution DNA Sizing with the Agilent Femto Pulse System

Accurately size high molecular weight DNA smears up to 165 kb in under 1.5 hours for generating large-insert SMRTbell libraries

The Agilent Femto Pulse system is a fast, high-resolution automated capillary electrophoresis (CE) platform that utilizes pulsed-field electrophoresis to separate high molecular weight DNA fragments. This platform allows important DNA quality checkpoints to be completed in less than 1.5 hours with minimal sample input for *de novo* large genome sequencing projects and other PacBio® applications leveraging multi-kilobase read lengths. The instrument can be used in place of gel-based pulsed-field electrophoresis (PFGE) systems to fully support generation of large-insert SMRTbell® libraries with accurate sizing to 165 kb. Alternative DNA sizing instruments cannot accurately resolve large DNA fragments in this range.

- Complete critical DNA quality checkpoints in under 1.5 hours
- Accurately size DNA fragments up to 165 kb
- Conserve sample with femtogram-level DNA input as low as 50 fg/μL for fragments and 5 pg/μL for genomic DNA smears
- Improve overall workflow efficiency for large-insert SMRTbell library preparation

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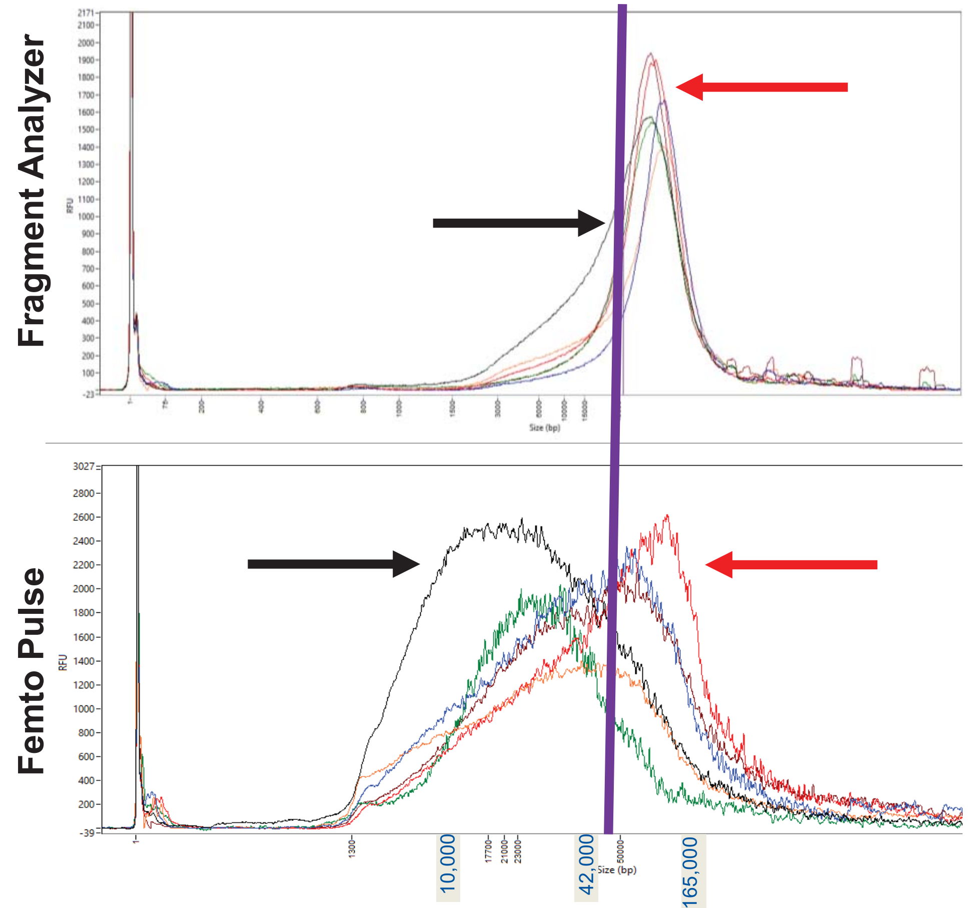
Genomic DNA Analysis

Agilent Femto Pulse system provides true size distribution



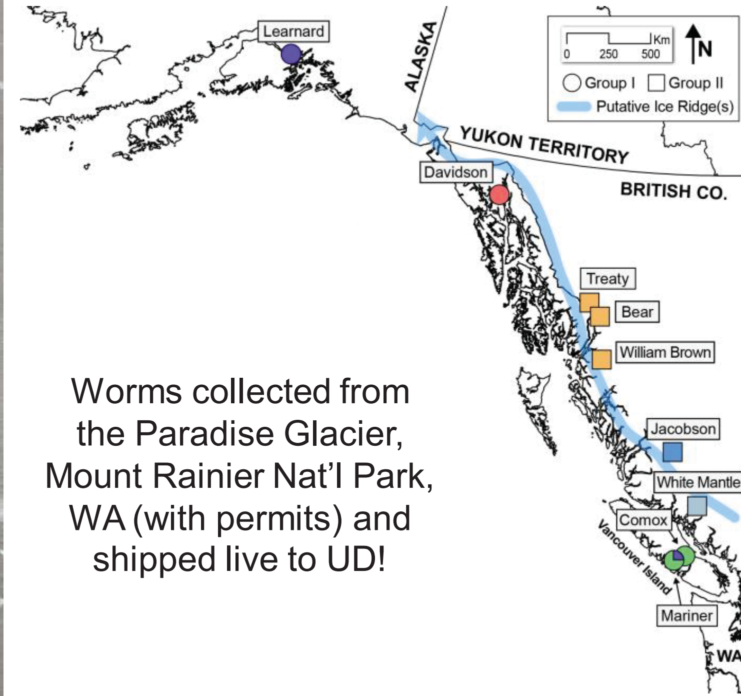
Pulsed-field electrophoresis prevents peak compression seen on direct field instruments.

Genomic DNA 165 kb kit (FP-1002)

Quantitative DNA		Qualitative DNA	Quantitative RNA
Genomic DNA 165 kb 1.3 bp to 165 kb	**US NGS** 100 bp to 6,000 bp	**55 kb BAC** 75 bp to 48.5 kb	**US RNA** 200 nt to 6,000 nt
FP-1002	FP-1101	FP-1003	FP-1201
Smear 5 pg/µL – 500 pg/µL	Smear 25 pg/µL – 250 pg/µL	Single Fragment 1 pg/µL – 25 pg/µL	15 pg/µL – 250 pg/µL
Fragment 0.3 pg/µL – 30 pg/µL	Fragment 0.1 pg/µL – 5 pg/µL	Max input 100 pg/µL	
	Fragment LOD 50 fg/µL		LOD 2.5 pg/µL
Genomic Quality Number (GQN)			**RNA Quality Number (RQN)** **DV_{200}**

Worm size: Small (~1.5cm)

Genome size: Big (~1.5 Gb)



Worms collected from the Paradise Glacier, Mount Rainier Nat'l Park, WA (with permits) and shipped live to UD!

Hotaling *et al.*, (2019) *Proc. Royal Soc. B*

Procedure & Checklist - Preparing SMRTbell® Libraries Using Express Template Preparation Kit v2.0 With Low-Input DNA

This document describes preparing SMRTbell libraries from genomic DNA (gDNA) as low as 150 ng using SMRTbell Express Template Prep Kit v2.0 . The Express Template Prep Kit v2 is an "addition-only" workflow, which minimizes DNA loss during library construction, enabling library construction from low amounts of input DNA.

Fast, High-Resolution DNA Sizing with the Agilent Femto Pulse System

Accurately size high molecular weight DNA smears up to 165 kb in under 1.5 hours for generating large-insert SMRTbell libraries

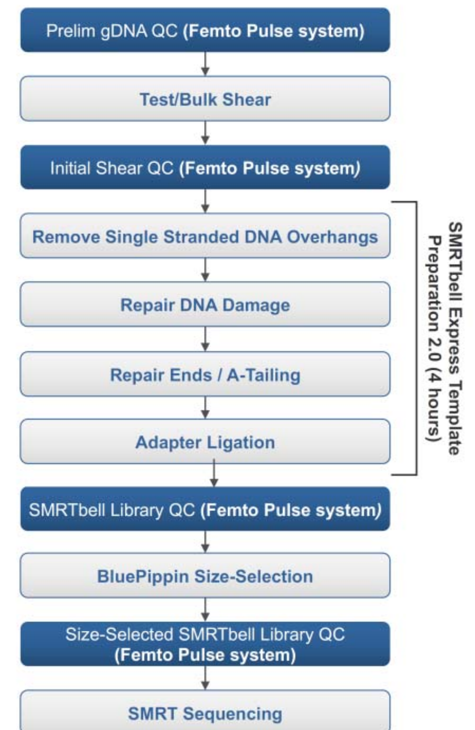


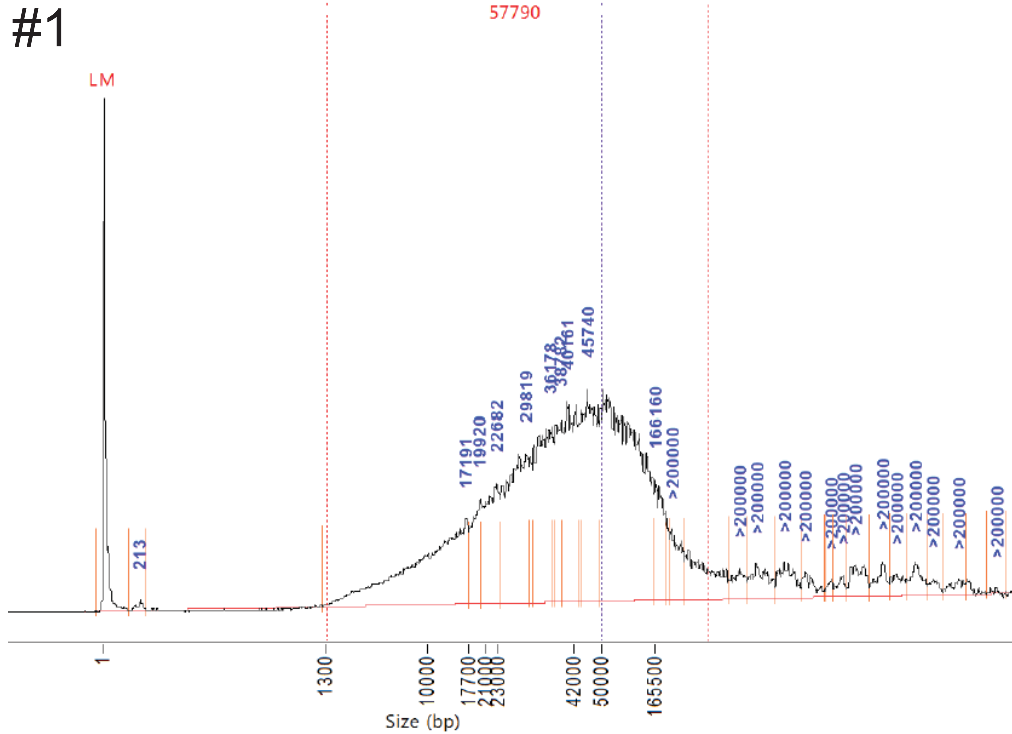
Figure 1. Large-insert library construction workflow with SMRTbell Express Template Prep Kit 2.0. *Sizing with the Agilent Femto Pulse system replaces all PFGE to complete critical QC checkpoints in less than 1.5 hours (blue boxes).*

DNA Isolation

- Worm was removed from ice and placed in 1.5ml tube containing lysis solution
  - Qiagen's MagAttract HMW DNA kit (modified protocol)
- Polypropylene micro-pestle was inserted and raised and lowered 5-10 times
- Lysis was carried out at 56°C for 30 minutes
- All mixer steps were replaced with rotations
  - Takes a bit longer but we see improved size
- Performed 2 elutions with elution buffer heated to 37°C

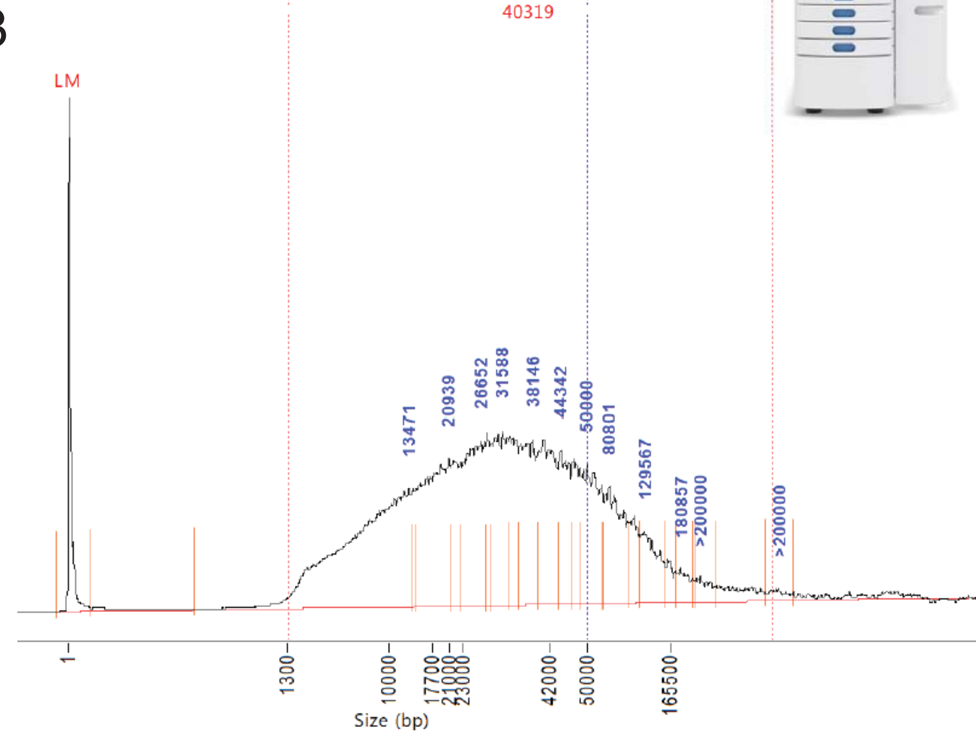
2 worms, 2 isolations

#1



Yield: 750 ng

#3



Yield: 500 ng

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RFU
6038
5900
5800
5700
5600
5500
5400
5300
5200
5100
5000
4876
LM
96294
1
1300
10000
17700
21000
23000
42000
50000
165500
Size (bp)

- Qubit dilutions can be used

2 isolations, 2 libraries

- Worm #1
  - 750ng of gDNA subjected to a 10kb cut on BluePippin
  - Only recovered ~100ng of size-selected DNA
  - Final library yield: 9ul of 4ng/ul SB; Avg. size ~32kb

- Worm #3
  - 500ng of gDNA
  - No size selection done on DNA before library prep
  - Library was cleaned utilizing a modified AmpPure protocol to remove fragments <3kb
  - Final library yield: 9ul of 25.5ng/ul SB; Avg. size ~29kb

Procedure & Checklist – Using AMPure® PB Bead for Removing < 3kb SMRTbell® Templates

Required Materials

Item	Vendor	Part Number
AMPure PB Beads	Pacific Biosciences	100-265-900
DNA Suspension Buffer, pH 8.0 (Tris 10 mM, EDTA 0.1 mM) 15 mL High-Clarity Polypropylene Conical Tube Wide Orifice Tips (RT-LTS-A-200 µL-/F/L/W-960/10)	Teknova Corning Rainin	T0227 352096 30389241

Dilute AMPure PB Bead Solution with DNA Suspension Buffer to 40%

The final AMPure PB bead concentration is critical to the success of this procedure. Therefore, accurate pipetting is of utmost importance to achieve a final 40% AMPure in DNA Suspension Buffer.

Reagent	Volume	✓	Notes
DNA Suspension Buffer	3.0 mL		
AMPure PB Bead (stock reagent)	2.0 mL		
Total Volume	5.0 mL		

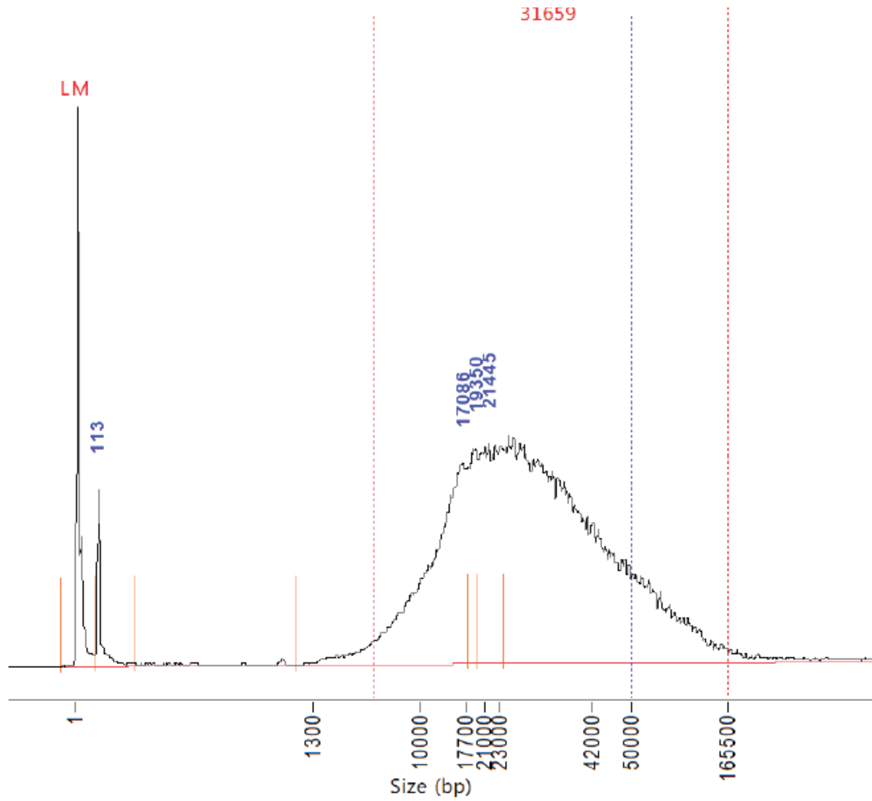
1. Bring AMPure PB beads stock to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 3.0 mL of the DNA Suspension Buffer into a 15 mL conical tube.
4. Add 2.0 mL of the stock AMPure PB bead to the 3.0 mL DNA Suspension Buffer. When pipetting the viscous AMPure PB bead solution, pipette slowly to ensure volume is as precise as possible. Large residual AMPure solution adhering to the tip should be removed prior to addition to the 3mL Suspension Buffer.
5. Vortex the diluted AMPure PB bead solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 weeks for future use.

Customer Collaboration

- 1:2.5 dilution of stock AmpPure PB beads
- Clean the library with 2.2X diluted beads

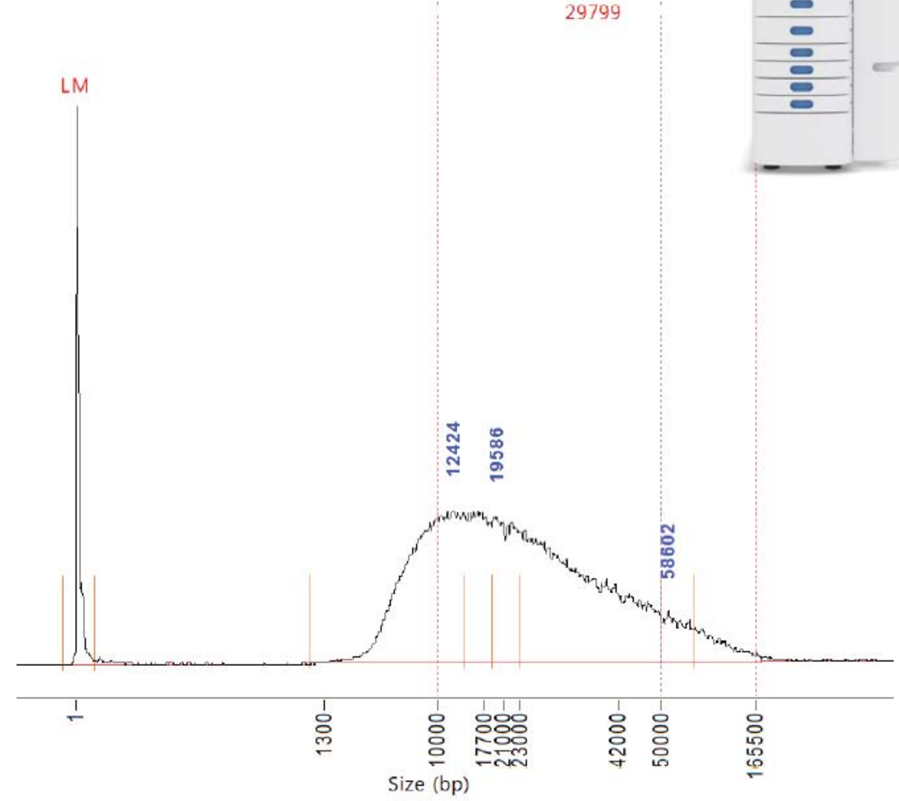
Final SMRTbell Libraries

#1



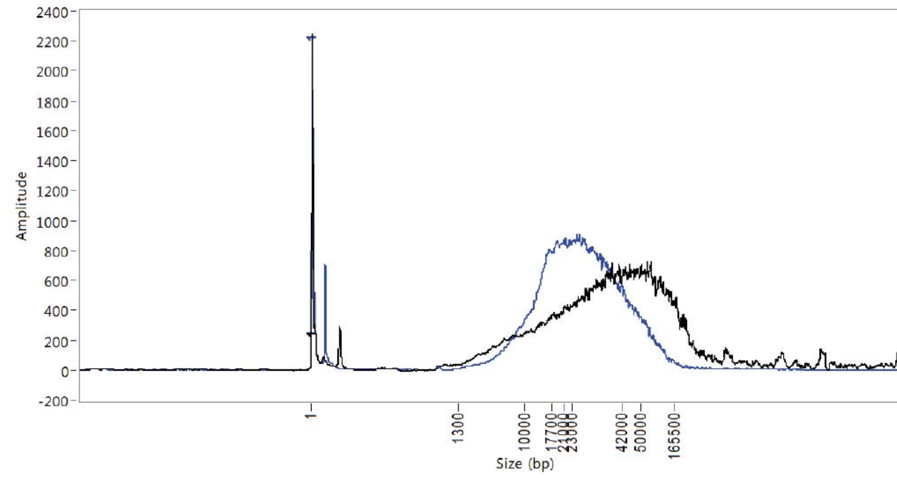
Yield: 9ul of 4.0ng/ul

#3

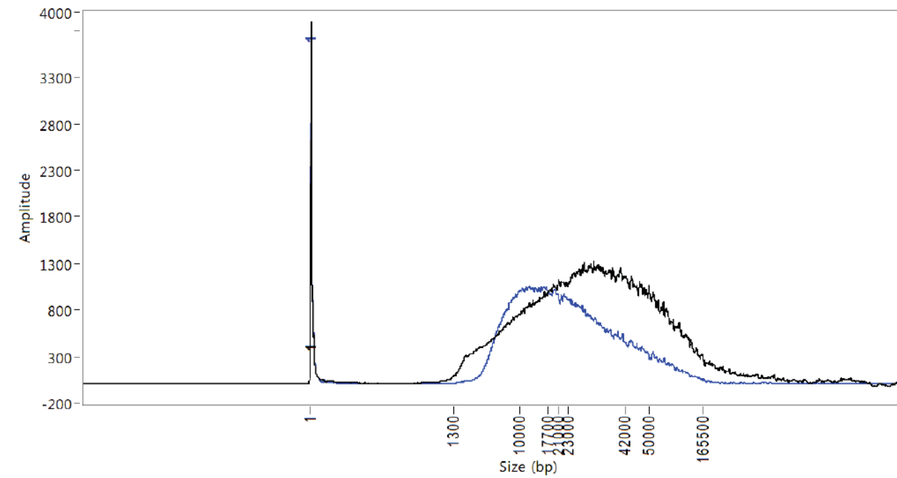


Yield: 9ul of 25.5ng/ul

Worm 1
gDNA and Final SB



Worm 3
gDNA and Final SB



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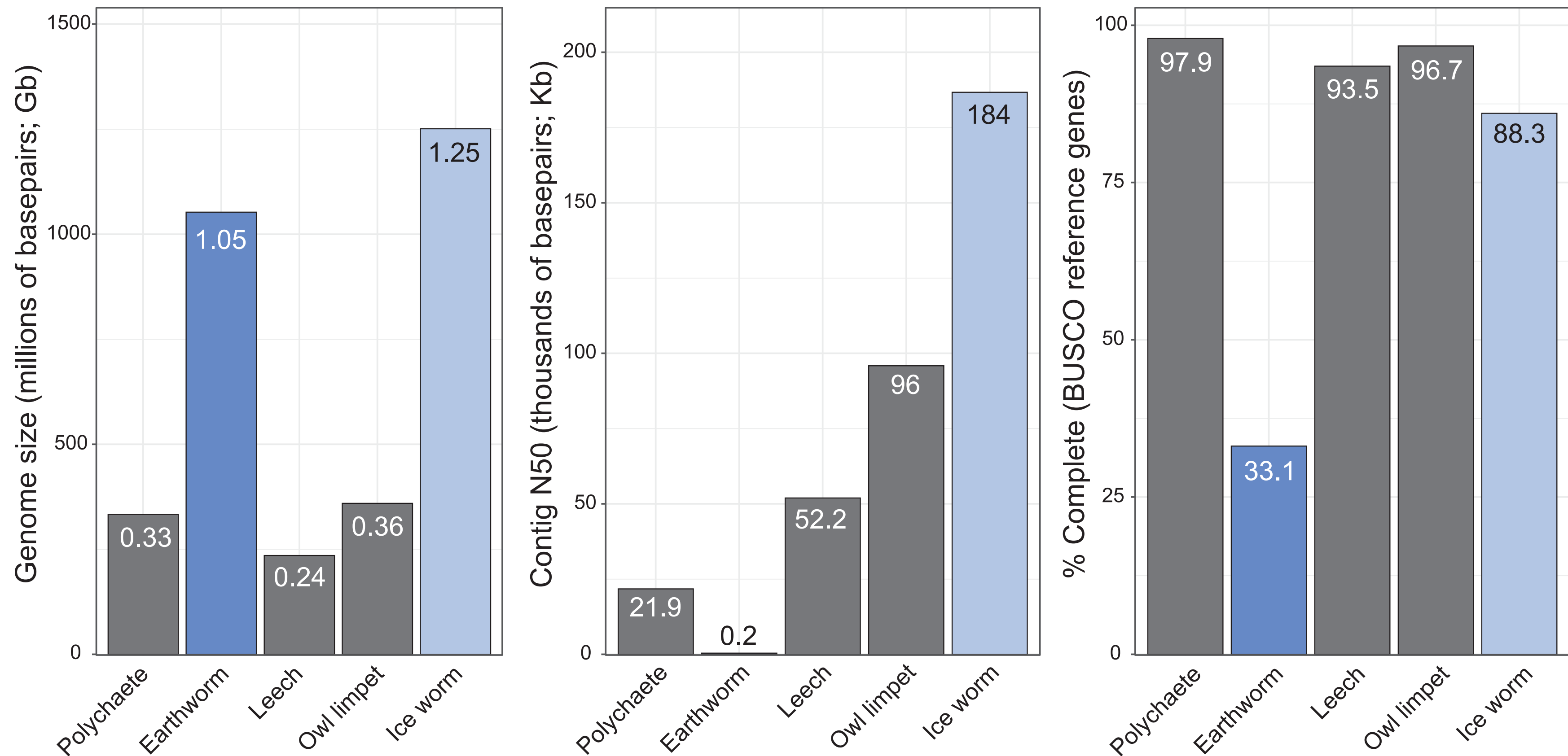
Worm	SMRT cell	Movie Time (hrs)	PE (hrs)	On-plate Conc. (pM)	P1 (%)	Yield (GB)	Mean Poly. Read Length (kb)	Insert N50 (kb)
3	1M v3	10	2	4	54.1	14.1	22.08	10.53
3	1M v3 LR	15	0	4	55.3	14.7	26.52	13.41
1	1M v3 LR	15	0	4	6.4	0.89	22.15	18.87

- Worm 3: SB cleaned with modified AmpPure protocol
- Worm 1: gDNA subjected to 10kb cut on BluePippin

Total of 10 cells

Ice worms	1
Low-input libraries	1
SMRT cells	10
Mean Poly. Read Length (kb)	21.6
Insert N50 (kb)	12.75
Total yield (Gb)	159
Unique mol. yield (Gb)	59.6



- **Genome assembly done with FALCON**

SO, IT'S MORE CONTIGUOUS, DOES THAT MATTER?

AMP Deaminase:

- Key regulator of energy metabolism.
- Ice worms maintain atypically high energy levels in the face of cold[1].
- Partial sequence (540 a.a.) available for ice worms[1].

Research Article

Divergence of AMP Deaminase in the Ice Worm *Mesenchytraeus solifugus* (Annelida, Clitellata, Enchytraeidae)

Roberto Marotta,[1] Bradley R. Parry,[2] and Daniel H. Shain[2]

[1] Department of Biology, University of Milano, via Celoria 26, 20133 Milano, Italy
[2] Department of Biology, Rutgers The State University of New Jersey, 315 Penn Street, Science Building, Camden, NJ 08102, USA

	Ice worm (*M. solifugus*) PacBio assembly	Earthworm (*Eisenia fetida*) NGS Assembly (2019)[2]
Location	Contig 385 (372 kb)	Scaffold 182195 (12.2 kb)
Alignment	**Complete!** ☺	**Incomplete** 48.3% of the protein is missing (20 a.a. near N-terminus, 241 a.a. of C-terminus)

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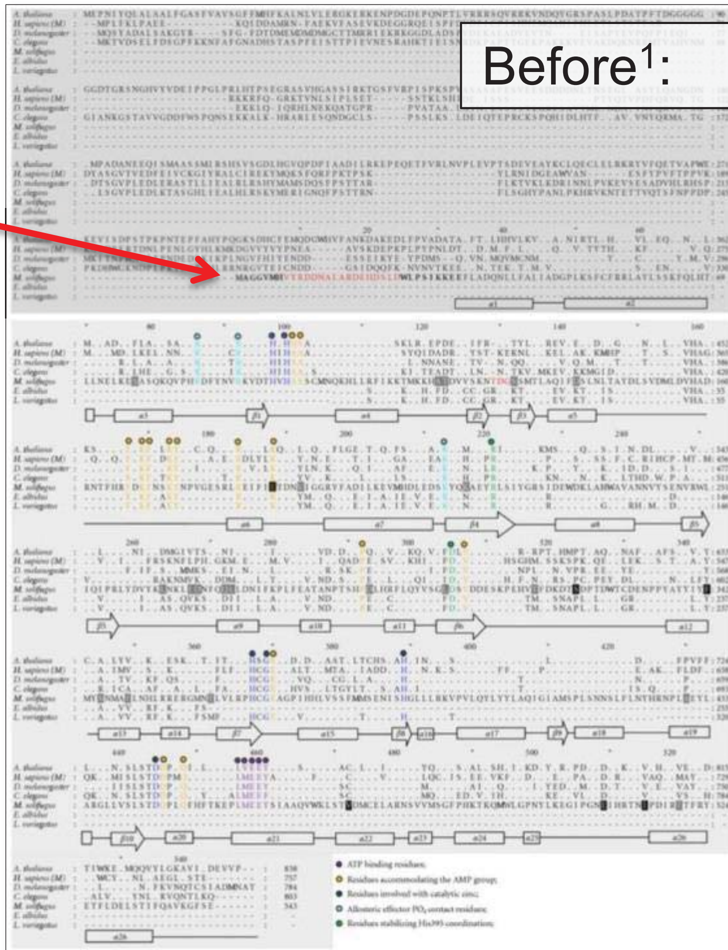
[1] Marotta et al. (2009) Intl J Evol Bio, doi:10.4061/2009/715086; GenBank Accession #ACF35336.1
[2] GCA_003999395.1, CSIR Insitute of Genomics and Integrative Biology, 2019/01/04
Slide courtesy of Scott Hotaling

Start of alignment, N-terminus missing



[1] Marotta et al. (2009) Intl J Evol Bio, doi:10.4061/2009/715086
Slide courtesy of Scott Hotaling

Conclusions

- Library construction from as little as 100ng is possible
  - Depending on the size of the genome and the desired coverage this may be enough but not in this case.

- Sequenced 10 cells, which generated 159Gb of data (60Gb > 10kb)
  - We did not sequence the entire library prep (16-17 cells were possible)

- Obtaining long reads from one individual led to an improved assembly
  - More work to be done
  - Contamination IS an issue

Agilent Femto Pulse Field Promotion for PacBio Customers

Eliminate Overnight PFGE
with the Agilent Femto Pulse system

Start Sequencing Large-Insert SMRTbell Libraries Sooner

Accurate quality measurements of large DNA fragments are needed to optimize project outcomes and maximize sample recovery with long-read sequencing. The Femto Pulse system replaces overnight pulsed-field gel electrophoresis (PFGE) to complete critical quality control checkpoints in about 1.5 hours.

This system allows important DNA quality measurements to be completed with minimal sample input for *de novo* large genome sequencing projects and other PacBio applications leveraging multi-kilobase read lengths.

Improve your overall workflow efficiency for large-insert SMRTbell library preparation with:

- Large fragment separation up to 165 kb
- Picogram level sample consumption
- Simple sample preparation
- Fast electrophoresis times

PacBio customers receive 20% off Femto Pulse System + one free Genomic DNA 165kb kit.

PacBio Certified Service Providers receive 10% off Femto Pulse Analysis kits purchase.

For more information, please contact your Genomics Sales Representative: genomics-sales@agilent.com

Promotions valid until October 18, 2019.

Reference code: **DGG_1906_SUMPRO_SP_AF-05**

For product information, visit: **www.agilent.com/genomics/femto-pulse**

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PacBio customers receive 20% off Femto Pulse System + one free Genomic DNA 165kb kit.

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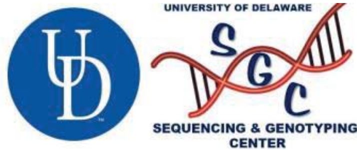

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