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Direct oligonucleotide sequencing with nanopores

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ABSTRACT

Third-generation DNA sequencing has enabled users to sequence long, unamplified DNA fragments with minimal steps. Direct sequencing of ssDNA or RNA gives valuable insights like base-level modifications, an estimate of phosphoramidite synthesis yield and analysis of strand quality, without the need to add the complimentary strand. Direct sequencing of single-stranded nucleic acid species is challenging as they are non-compatible to the double-stranded sequencing adapters used by the manufacturers. The MinION platform from Oxford Nanopore Technologies performs sequencing by passing single-strands of DNA through a layer of biological nanopore sensors, although the sequencing is performed on single-strands of DNA, the recommended template by the instrument manufacturer is still double-stranded. We have identified that the MinION platform from Oxford Nanopore can perform sequencing of short, single-strand oligonucleotides directly without amplification or second-strand synthesis by performing a single annealing step before library preparation. Short 5’ phosphorylated oligos when annealed to an adapter sequence can be directly sequenced in the 5' to 3' direction via nanopores. Adapter sequences were designed to bind to the 5’ end of the oligos and to leave a 3’ adenosine overhang after binding to their target. The 3’ adenosine overhang of the adapter and the terminal phosphate makes the 5’ end of the oligo analogous to an end-prepared dsDNA, rendering it compatible with ligation-based library preparation for sequencing. An oligo-pool containing 42,000, 120 nt orthogonal sequences were phosphorylated and sequenced using this method and ~90% of these sequences were recovered with high accuracy using BLAST. In the nanopore raw data, we have identified that empty signals can be wrongly identified as a valid read by the MinION platform and sometimes multiple signals containing several strands can be fused into a single raw sequence file due to segmentation faults in the software. This direct oligonucleotide sequencing method enables novel applications in DNA data storage systems where short oligonucleotides function as the primary information carriers.

MATERIALS

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Oligonucleotides:

	A	B	C	D
	Oligo_ID	Sequence (5' --> 3')	Concentration	Procurement
	INS3	/5Phos/GCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGACGCAGCCCGCAGGCAGCCCCACACCCGCCGCCTCCTGCACC/3SpC3/	100 uM	IDT (Ultramer)
	INS3 RC	CCCGCCCAGCA	100 uM	IDT (Standard desalting
	EINS3	/5Phos/AGGCTTCTTCTACACACCCAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGTGAA/3Phos/	100 uM	IDT (Ultramer)
	EINS3 RC	CTTGGGTGTGTAGAAGAAGCCTA	100 uM	IDT (Standard desalting
	ArcFP_MoSS5	ACTGAGGTTGTAATCTGCGTTGTAGA	100 uM	IDT (Standard desalting
	3xr6 oligo-pool	See supplementary table	10 ng/ul	Twist (Oligo Pools)

Supplementary table

Enzymes and buffers:

T4 Polynucleotide Kinase - 500 units **New England Biolabs Catalog #M0201S**

Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog #M0367S**

TE pH 7.5 **IDT Technologies Catalog #11-01-02-02**

Qubit™ ssDNA Assay Kit **Thermo Fisher Scientific Catalog #Q10212**

Nuclease Free Water **IDT Technologies Catalog #11-04-02-01**

Wash kit:

Monarch® PCR & DNA Cleanup Kit (5 µg) **New England Biolabs Catalog #T1030**

Sequencing kit:

Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109**

Equipment:

Open qPCR (Single Channel) - ChaiBio - E013101
Microcentrifuge - Dlab - D3024
Qubit 4 - ThermoFisher - Q33226

Sequencer

MinION - Oxford Nanopore Technologies (ONT) - MIN-101B

Normalization

1 Normalize 3xr6 dried oligo-pool to 0.25 uM with TE buffer and verified with Qubit 4 using Qubit ssDNA Assay Kit

2 INS3 and EINS3 are diluted from their stock concentrations to 0.5 uM with TE buffer and verified with Qubit 4 using Qubit ssDNA Assay Kit

3 INS3 RC, EINS3 RC and ArcFP oligos are normalized by the supplier at 100 uM concentration and are diluted to 1 uM concentration.

Phosphorylation of 3xr6

50m

4 Mix the following reagents into a PCR tube.

	A	B
	Reagent	Volume µl
	3xr6 oligo-pool	8
	T4 PNK reaction buffer	2.5
	10 mM ATP	2.5
	T4 PNK	0.5
	Nuclease-free water (NFW)	11.5

5 Incubate at 37 °C for 00:30:00 30m

6 Heat inactivation at 65 °C for 00:20:00 20m

7 The mixture is washed using Monarch spin columns with the standard oligonucleotide cleanup protocol. The purified DNA is eluted into 9 ul of IDTE instead of the given Elution buffer.

Library preparation of INS3 32m

8 Create a triplicate of the following reaction mix:

	A	B
	Reagent	Volume µl
	0.5 uM INS3	0.5
	1 uM INS3 RC	0.5

	A	B
	NFW	2

9 The reaction mixtures are heated to 94 °C for 00:02:00 and gradually cooled to room temperature for annealing. 2m

10 5 µL of AMX from the ligation sequencing kit is added to each of the tubes.
5 µL of Blunt/TA master mix is added to each of the tubes.

11 Tubes are incubated at room temperature for 00:10:00 10m

12 Three new MinION flow cells are used to sequence each reaction mix of the triplicate and the sequencing is performed with the standard parameters for 00:20:00 20m

Library preparation of EINS3

32m

13 Create a triplicate of the following reaction mix:

	A	B
	Reagent	Volume µl
	0.5 uM EINS3	0.5
	1 uM EINS3 RC	0.5
	NFW	2

14 The reaction mixtures are heated to 94 °C for 00:02:00 and gradually cooled to room temperature for annealing. 2m

15 5 µL of AMX from the ligation sequencing kit is added to each of the tubes.
5 µL of Blunt/TA master mix is added to each of the tubes.

16 Tubes are incubated at room temperature for 00:10:00 . 10m

17 Three MinION flow cells from the INS3 run are used to sequence each reaction mix of the triplicate and the sequencing is performed with the standard parameters for 00:20:00 . 20m

Library preparation of 3XR6

4h 12m

18 Elution of the phosphorylation step is split 3-ways for triplicate sequencing runs. A triplicate of the following reactions is performed:

	A	B
	Reagent	Volume µl
	Elution of 3XR6 phosphorylation	3
	1 uM ArcFP	1

19 The reaction mixtures are heated to 94 °C for 00:02:00 and gradually cooled to room temperature for annealing. 2m

20 5 µL of AMX from the ligation sequencing kit is added to each of the tubes.

5 µL of Blunt/TA master mix is added to each of the tubes.

21 Tubes are incubated at room temperature for 00:10:00 . 10m

22 Three new MinION flow cells are used to sequence each reaction mix of the triplicate and the sequencing is performed with the standard parameters for 04:00:00 . 4h