Oxford NANOPORE Technologies

WORKFLOW: SNV CALLING AND PHASING

Calling and phasing single nucleotide variants in the human genome with long nanopore reads

With an estimated 8% of the human genome still unsequenced[1], our understanding of genetic variation has been limited. Compared to short-read technologies, nanopore sequencing lacks GC bias and does not require PCR, enabling wider access to the genome for variant calling.

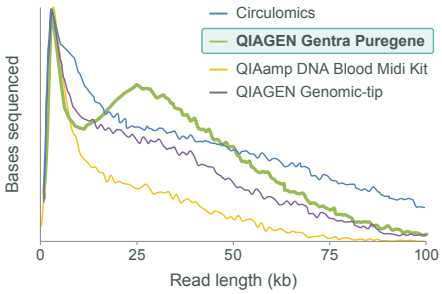
Assigning variants to the maternal or paternal chromosome (phasing) is important for understanding their inheritance and functional impact. Long and ultra-long nanopore sequencing reads greatly enhance phasing, as an individual read is more likely to contain multiple heterozygous single nucleotide variants (SNVs).

Here we present a simple workflow for calling and phasing SNVs in the human genome.

EXTRACTION: obtaining high molecular weight DNA

Find more extraction protocol recommendations for your sample type, plus guidance on DNA storage and contaminants: **community.nanoporetech.com/extraction_methods**

Long sequencing reads are required for optimal phasing. Obtaining high molecular weight (HMW) DNA from your sample is therefore crucial. Selecting the most suitable HMW DNA extraction method depends on your sample type. For DNA extraction from whole blood, we recommend the **QIAGEN Gentra Puregene Blood Kit**, which we have found maximises the production of long sequencing reads.



LIBRARY PREPARATION: selecting a kit

Find out more about size selection methods for long-read sequencing: **community.nanoporetech.com/extraction_methods**

We have found that read length has a strong effect on phasing performance. To prepare your extracted gDNA, we advise light shearing and size selecting for >10 kb fragments, for a read N50 of >32 kb. Alternatively, no shearing, with optional size-selection, for a read N50 of ≥25 kb, produces similar results (see Table). Internal testing has yielded good results using the **Circulomics Short Read Eliminator Kit** for size selection (to >10 kb), and the **Diagenode Megaruptor 3** for shearing.

To prepare gDNA for sequencing, we recommend the **Ligation Sequencing Kit (SQK-LSK109)**, providing the greatest throughput.

Read N50	Shearing method	Phase block N50**
11 kb	g-TUBE	80–200 kb
17 kb	SRE + MR-3*	150–300 kb
25 kb	**Unfragmented**	**1–2.5 Mb**
26 kb	SRE + MR-3	500–650 kb
32 kb	**SRE + MR-3**	**1–2.5 Mb**
36 kb	**SRE + MR-3**	**1.5–3 Mb**

*Circulomics Short Read Eliminator Kit + Diagenode Megaruptor 3
** Established using data for human chr21

Find out more about sample prep, including rapid and multiplexing options: **nanoporetech.com/products/kits**

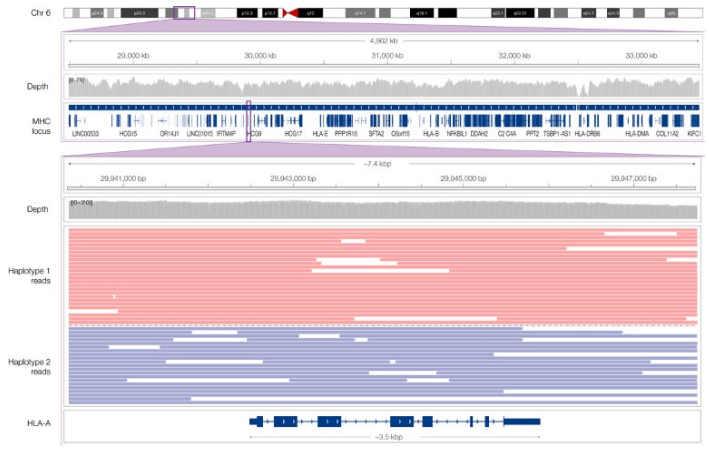
SEQUENCING: generating high yields of long reads with PromethION

Find out more about PromethION:
nanoporetech.com/products/promethion

For SNV calling, we recommend sequencing a human genome to 40x–60x read depth; this can be achieved by sequencing a single genomic sample on one or two PromethION™ Flow Cells for 72 hours.

Whilst longer reads significantly improve phasing, read depth is less important, with good metrics achieved at 45x and diminishing returns for higher depth.

We recommend basecalling in high accuracy mode with Guppy version 3.6.1 or higher. Throughput can be maximised by performing a nuclease flush and loading fresh library every 24 hours.



Find out about nanopore sequencing service providers: **nanoporetech.com/services/providers**

ANALYSIS: selecting tools for SNV calling & phasing

Find out more about data analysis solutions:
nanoporetech.com/nanopore-sequencing-data-analysis

To call SNVs in the human genome we recommend Medaka[2], developed and supported by Oxford Nanopore. This tool is ideal when turnaround time is of high importance: runtime is 100 hours with 32 CPU cores, and can be reduced to <48 hours using a single PromethION or GridION™ GPU and 8 CPU cores.

Alternatively, we have found that the third-party PEPPER/DeepVariant[3] workflow provides the best SNV calling metrics for datasets with read depths <60x, basecalled with Guppy 3.6.1 and above, although this method requires a longer runtime.

We advise the third-party tool WhatsHap[4] for phasing, alongside PEPPER/DeepVariant. WhatsHap is integrated into Medaka, so this additional step is unnecessary when using Medaka for SNV calling.

FASTQ input file

Alignment	minimap2
SNV calling	DeepVariant or Medaka
Phasing	WhatsHap

VCF output file

Find out more at: **nanoporetech.com/applications/investigation/snvs-phasing**

Oxford NANOPORE Technologies

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References:
1. Miga, K.H., Eisenhart, C., and Kent, W.J. Utilizing mapping targets of sequences underrepresented in the reference assembly to reduce false positive alignments. *Nucleic Acids Res.* 43(20):e133-e133 (2015).
2. © 2020 Oxford Nanopore Technologies Ltd.
3. Shafin, K. PEPPER. Software. Available at: https://github.com/kishwarshafin/pepper/blob/master/docs/PEPPER_variant_calling.md [Accessed: 16 July 2020].
4. Martin, M. WhatsHap: fast and accurate read-based phasing. *bioRxiv*. DOI: https://doi.org/10.1101/085050 (2016).