Nanopore Sequencing Provides Robust and Reliable Allele and Epitope Level HLA Typing for Deceased Donors in Less than 6 hours.

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INTRODUCTION

- Next-generation sequencing (NGS) has been widely adopted for clinical HLA typing and advanced immunogenetics researches.
- However there are limitations with NGS approaches, as the short sequences produced (~150-600bp) can limit the accurate alignment of reads to reference allele, impairing correct assignment and phasing of the alleles.
- Third-generation, or Single Molecule Real-Time (SMRT) sequencing technologies are ushering in a new era of sequencing.
- Platforms such as PacBio (Menlo Park, CA) and Oxford Nanopore Technologies (Oxford, UK) can sequence long stretches of DNA (the longest being 4million bases), without the need to fragment the DNA prior to reading the sequence.
- Here we present the results of our proof of concept Nanopore-based long read PCR solution for HLA genotyping.
- And further develop it into a high-resolution HLA typing deceased donor workup protocol from sample to epitope level matching in ~6hrs.

METHODS AND MATERIALS

- DNA from 50 patients (25 donors and recipients) representing a 6 month period of donor workups were selected for this study.
- All samples had been previously typed using current on-call LinkSeq RT-PCR (Linkage BioScience) and retrospective Holotype v3 NGS (Omixon) typing.
- Samples were amplified using the Omixon Omnitype 11 loci single tube assay (full gene for HLA-A, -B, -C, -DQA, -DPA and partial genes for DRB1, DRB3/4/5, DQB1 and DPB1).
- Amplicons were then prepared using the 1D ligation kit (SQK-LSK109), and run on a standard R9.4 flow cell.
- Each flow cell was run for between 1-6 hours total sequencing time. Raw FAST5 files were live basecalled using the MinKNOW GUI using both the high accuracy mode.
- HLA allele assignment was performed blind by our collaborators and the Vancouver team.
- Two field typings were then input to HLA Matchmaker v3 for each Donor/Recipient pair.

RESULTS

- HLA allele assignment performed blind, with no manual editing.
  - 3rd or 4th field typing achieved for 100% of alleles
  - Concordance was only assessed to 2 field typing

HLA assignments overall

2112 alleles total	864 cls1	18 discrepant (2nd field)	98% correct assignment
	1248 cls2	67 discrepant (2nd field)	95% correct assignment

*some of the 2nd field discrepancies resulted in a G group member

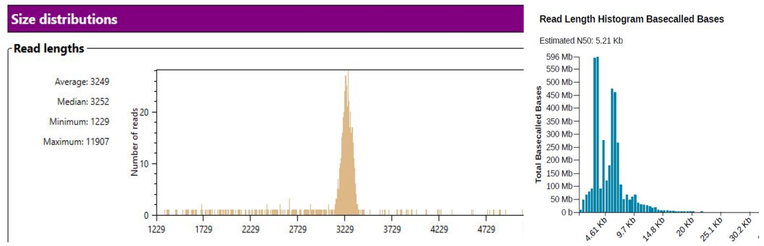


Fig 1. The average Miseq fragment size was ~400bases, compared to the average ONT fragment size of ~4000bases.

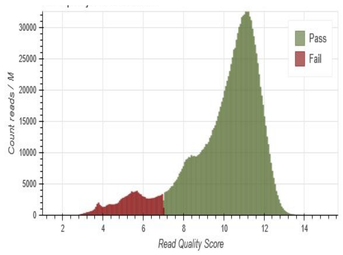


Fig 2. Average Qscore for reads from MiSeq run *~34 (data not shown)* compared to average Qscore for reads from ONT run ~15.

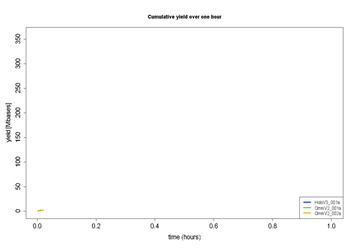
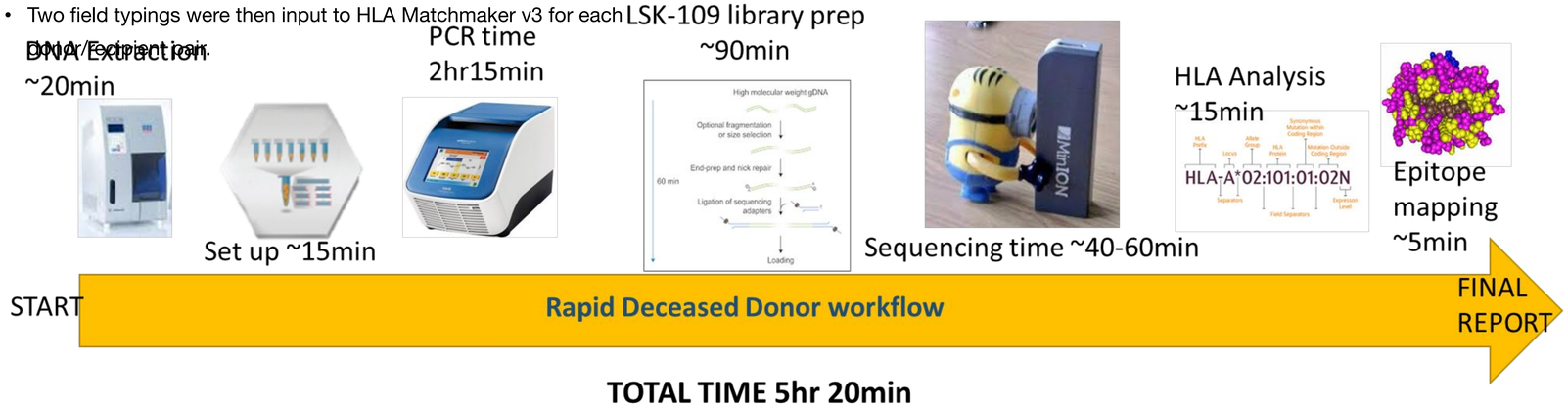


Fig 3. Average data yield from MiSeq ~5000Mb/*20hr (not shown)* compared to average data yield from ONT ~500Mb/*1hr*



FURTHER IMPROVEMENTS

- The use of nanopore technologies in immunogenetics is fast becoming a reality.
- Clinical utility of this platform for HLA genotyping is still improving and includes modifications at every stage of the process.
  - **Library preparation**
    - The Omixon Omnitype protocol will reduce amplification time from the current 2hr15 to **1hr45.**
    - We are also evaluating the use of a hybrid capture approach instead of PCR amplification of the HLA targets. With no PCR amplification step, the workflow time would be significantly less and more streamlined.
  - **Flowcell updated**
    - Validation started already. We have run our first 24 samples on a new R10.3 flow cell.
    - These flow cells from ONT contain a double k-mer recognision site which should increase accuracy through homopolymers
    - We are also validating the Flongle flow cell version, which is ONT's smallest and cheapest capacity flow cell. For deceased donor typings, where you only want to type a single patient, this will be the flow cell recommended for use.
  - **Bioinformatics**
    - Mk1c device is ONT's al-in-one sequencer and computer. The use of this device for on-call donor workup will be critical as it is a single flow cell device, with high GPU power, allowing for faster basecalling.
    - We are currently comparing the Fast basecalling vs High Accuracy basecalling algorithms, which are run immediately and in real-time, as the sequencing is read through the pore. For deceased donor workup, when time is of the essence, we may choose to run Fast basecalling.
    - HLA genotyping algorithms for alignment of long read sequences are still improving. We are continuing to collaborate with our development teams to further improve genotyping software performance.

CONCLUSION

- Whilst technological advancement has been able to improve the limitations of NGS sequencing, there are still nanopore bioinformatic improvements that need to be validated for HLA genotyping.
- The value of having long reads for HLA genotyping will be with the alignment and thus accurate assignment of high resolution HLA genes, including alignment outside of key HLA exons.
- However, here we present no only the ability to accurately HLA type using a nanopore technology, but also to do so in a timeframe suitable for deceased donor workup.
- The ability to perform high resolution typing for all donors and recipients would allow us to carry out epitope level matching for all renal transplants.
- This would have major clinical benefits, in particular for highly sensitized