

A Single Centre Experience of HLA Typing by TGS Using NanoTYPE™ assays

Kamla Reddi
Clinical Transplantation Laboratory
Guy's Hospital



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Next and Third Generation Sequencing (NGS/TGS)

- Historically HLA typing to second field was performed using the Sanger sequencing methods, achieving third field extremely laborious and difficult
- Limitations of Sanger sequencing : ambiguities due to polymorphisms in un-sequenced regions of HLA genes or due to cis/trans variants
- In 2000 the first NGS technology was launched and has since evolved with many different platforms now readily available
- NGS: high throughput, faster TaTs, increased accuracy, fewer ambiguities, can do multiple samples at once
 - but generally the methods used small reads joined together using bioinformatic algorithms which could lead to low coverage and ambiguities
- Omixon NanoTYPE™ (ONT) uses third generation sequencing which utilises long read sequencing
- Has a simplified workflow compared to short-read sequencing
- ONT uses nanopore sequencing methods that identifies the sequence of nucleotides by passing individual molecules through these pores and measures the changes in electrical current.

Equipment

- VeritiPro Thermal Cycler
- Qubit fluorometer
- Magnetic stand
- 96 Well cooler rack, 1.5ml tube cooler rack or ice bucket
- Microplate and Microtube centrifuge
- Vortex
- Adjustable volume pipettes, 8 channel adjustable volume pipettes (1.0-1000µl)
- Timer
- ONT MinION Mk1b
- ONT Flow cells (R9.4.1)
- Laptop/PC compatible/installed with MinKNOW software, NanoTYPE workbook (Excel) and NanoTYPER™ analysis software

Reagents/Consumables and Laboratory requirements

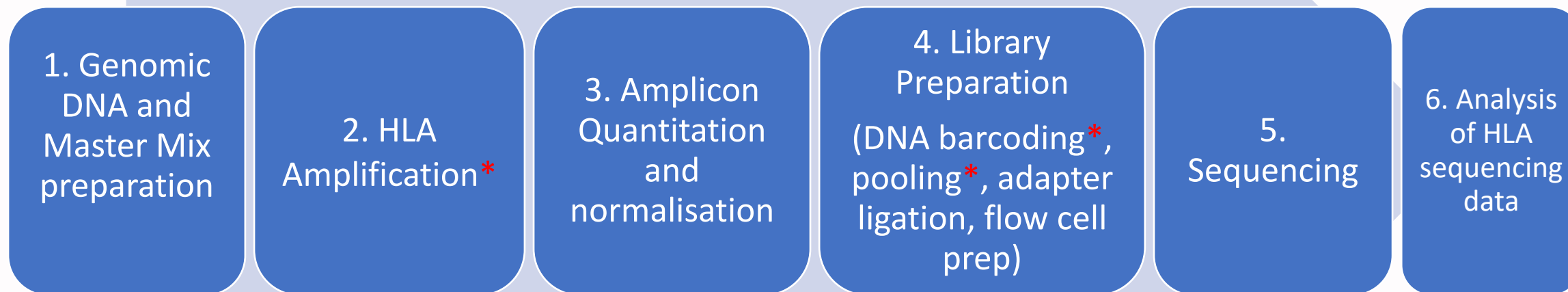
Reagents

- Omixon NanoTYPE® 24/11v2 RUO HLA Amplifications Kits
- Qubit dsDNA Broad Range (BR) Assay Kits
- Rapid Barcoding Kits
- ONT Flow Cell Wash Kits (each kits can perform 6 washes, contains wash mix, diluent and storage buffer)
- Molecular grade absolute ethanol
- Molecular grade water

Consumables: 1.5ml microcentrifuge tubes, 96-well plates, plate seals and pipette tips

Laboratory: Designated areas for Pre-PCR and Post-PCR to avoid contamination, and a good internet connection

Workflow



1 sample DNA to sequencer ~3hrs and 40 minutes, **DNA to results ~5 hours**

12 sample DNA to sequencer ~3hrs and 40 minutes, **DNA to results ~ 16 hours**

24 samples **DNA to results ~ 24 hours**

- Safe stopping, samples can be stored at 4°C overnight/1 month or -20°C for up to 3 months. Check manufactures recommendations

Amplification using the NanoTYPE™ Kits

- Able to perform all 11 loci (HLA-A, B, C, DQA1, DQB1, DPA1, DPB1, DRB1, DRB3,4,5)
- Each kit contains (able to amp 24 samples)
 - HLA Muti Primer Mix (diluted before use)
 - PCR Buffer
 - dNTP Mixture

DNA samples are diluted to predefined concentrations (10µl) using the NanoTYPE workbook before addition of PCR Master Mix (15µl) to an amplification plate.

The recommended minimum concentration of DNA required is 20ng/µl

The amplification plate is run on the thermocycler: 1 cycle at 94°C for 1 minute, followed by 30 cycles of denaturation at 98°C for 10 minutes and elongation at 68°C for 4.5 minutes

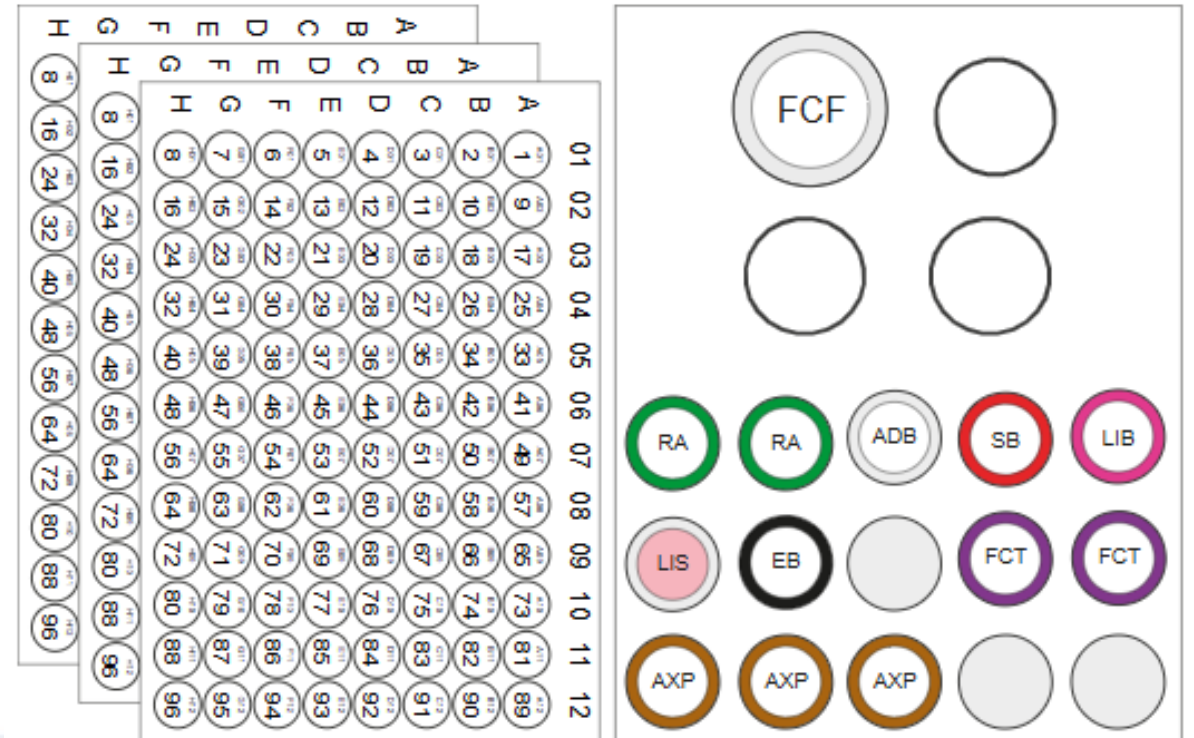


Rapid Barcoding

Post amplification, the products were quantified using Qubit BR assay, 200ng of amplicons were barcoded using the Barcoding kit. The PCR cycle consisted of a preset lid temperature of 105°C, followed by 30°C for 1 minute and 80°C for 1 minute

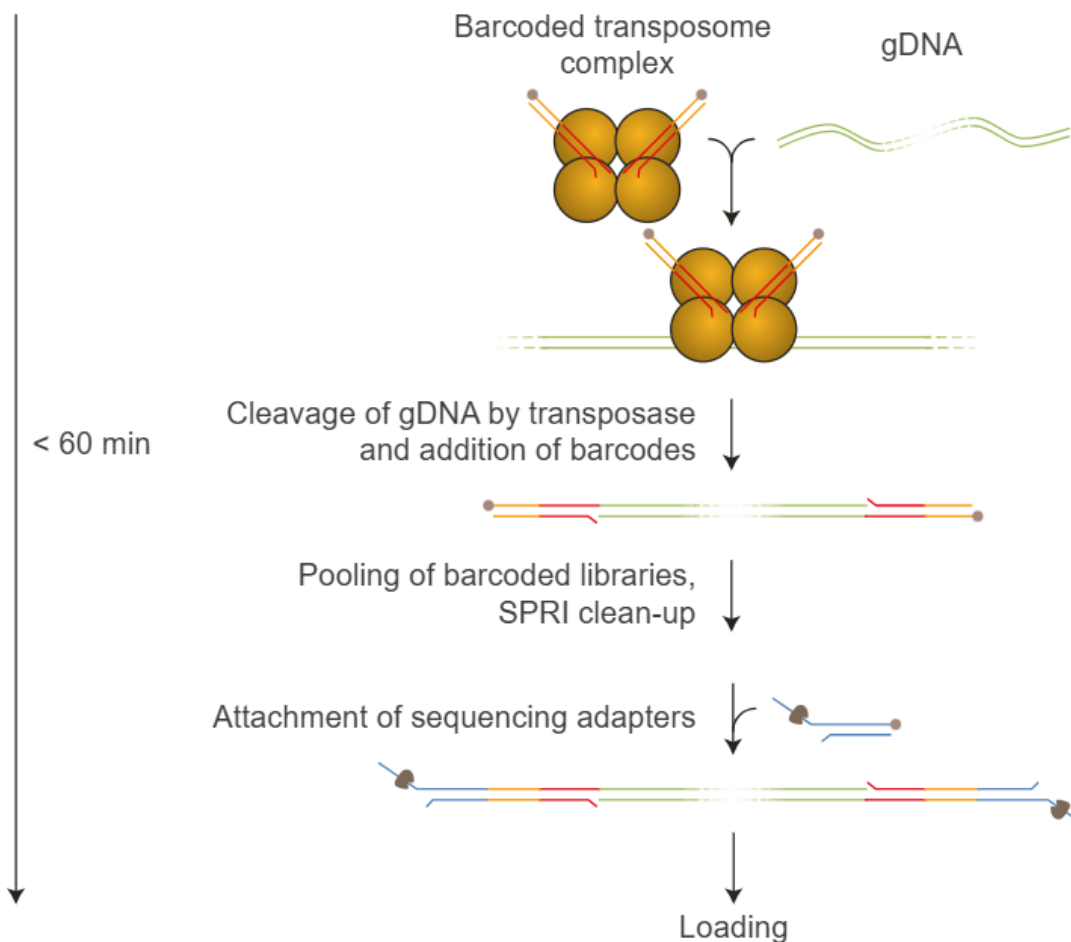
Rapid Barcoding kit (SQK-RBK114.96 from ONT)

- FCF: Flow cell Flush (Flush buffer, pre-aliquoted, sensitive to freeze/thaw)
- RA: Rapid Adapter
- ABD: Adapter Buffer
- SB- Sequencing buffer
- LIB: Library Beads (Loading beads)
- LIS: Library solution (Loading solution)
- EB: Elution Buffer
- FCT: Flow cell Tether
- AXP: AMPure XP Beads



Library preparation

- Samples were put on ice and pooled
- For 1-3 samples the use of AMPure XP bead clean up can be omitted
- For more than 4 samples the AMPure XP bead clean up process should be used.
- AMPure XP Beads and pooled library are mixed and incubated at room temperature, followed by elution using elution buffer
- Following this, sequencing adapters are attached
- Library is nearly ready for loading onto the flow cell and is kept on ice

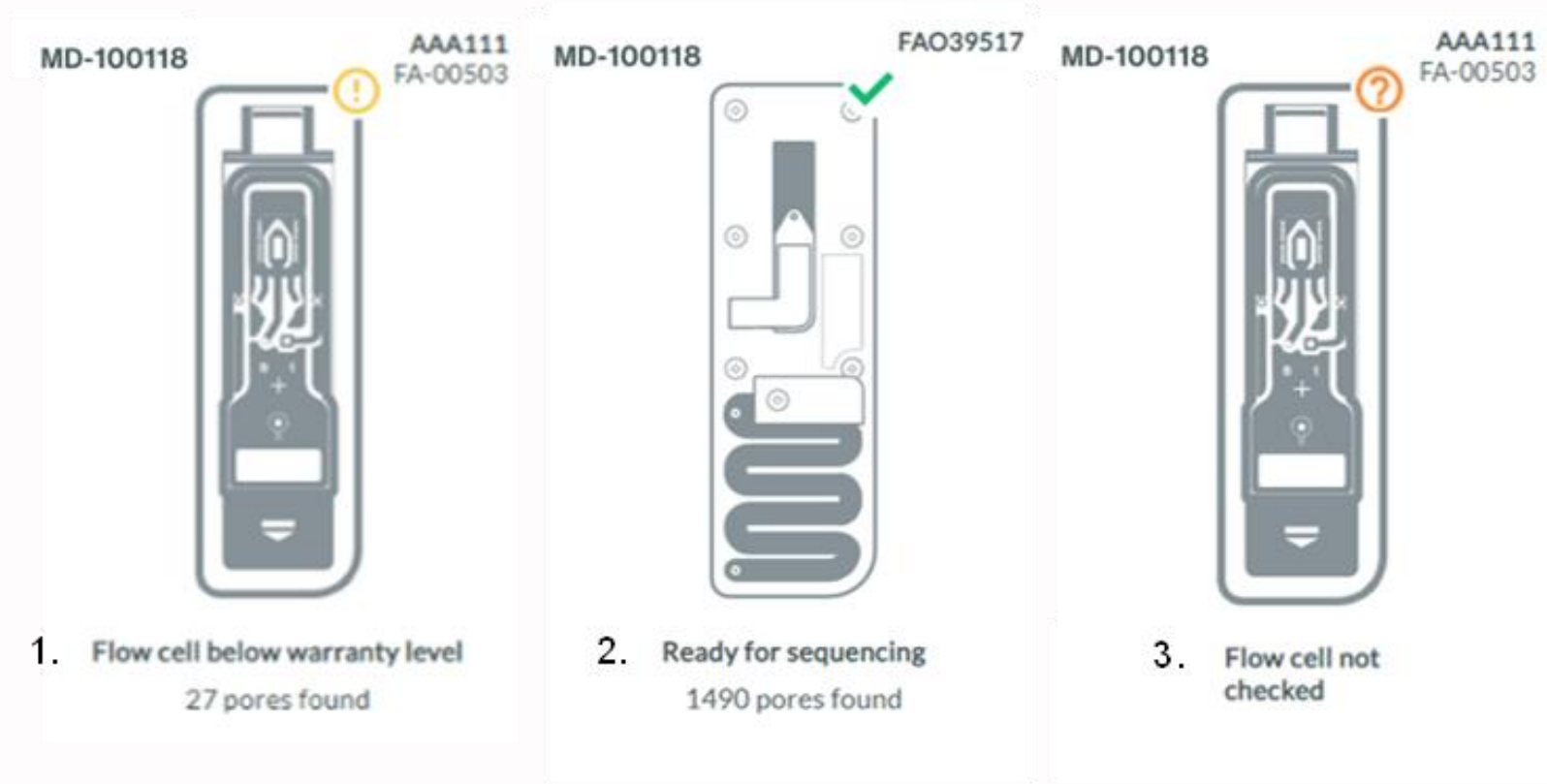


The MinION device

- Portal, takes up very little bench space
- Sequencing is performed using the MinKNOW software
- Sequences between temperatures of +18-24°C
- The flow cell needs to be checked using the MinKNOW software prior to sequencing for an estimated number of active pores available.
- A minimum of 800 pores is required before sequencing (warranty).
- Runs with up to 24 samples needs at least 800 pores.
- The more samples you have the more pores you need.

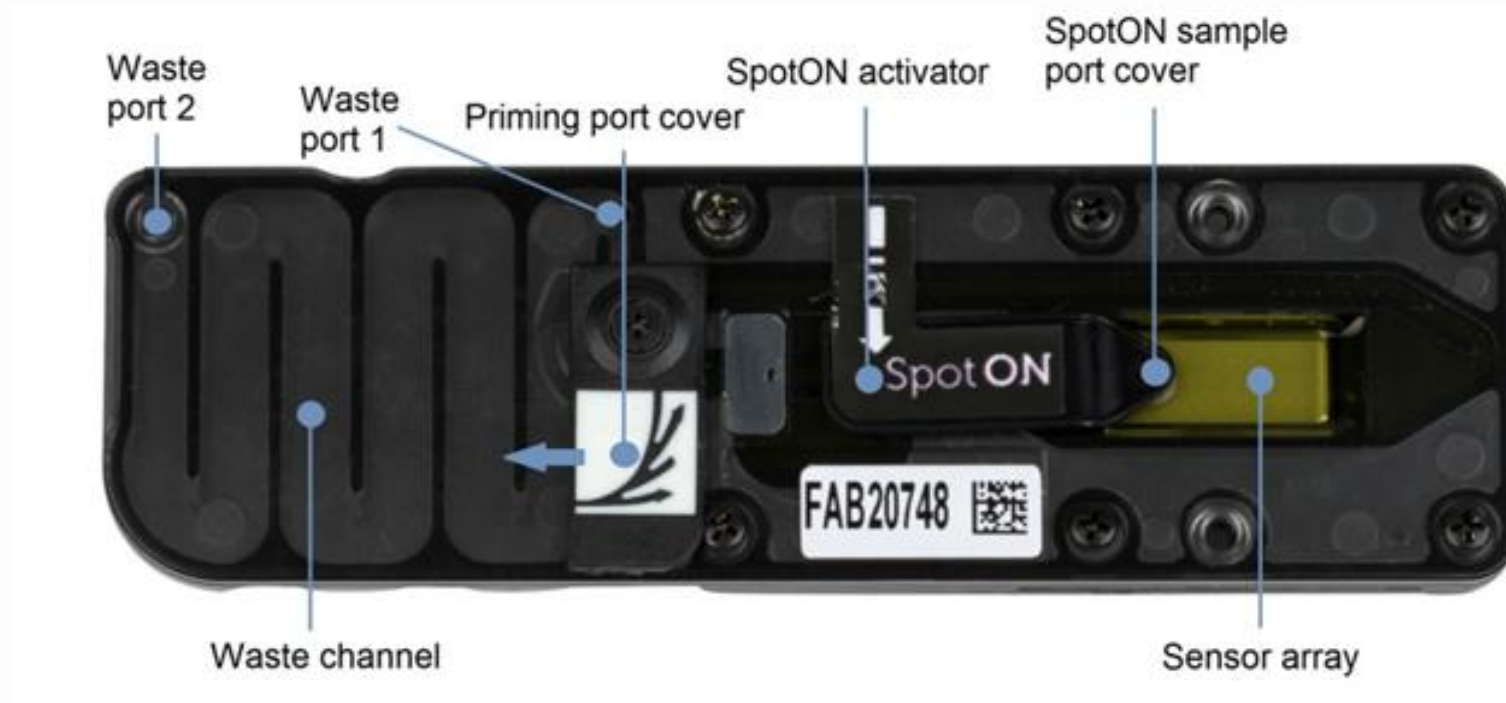


The Flow Cell Check



Flow Cell Priming and Loading

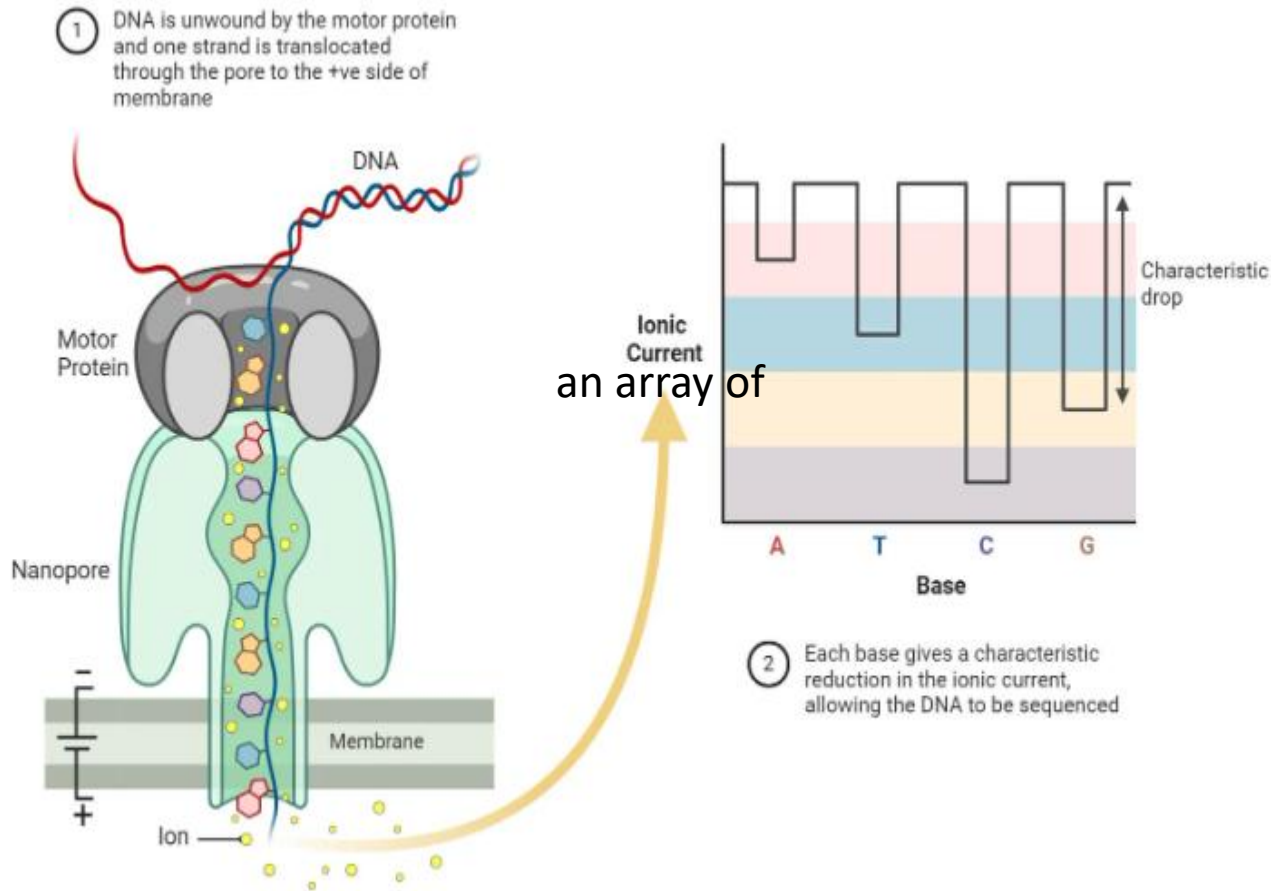
- Once the flow cell has passed its checks, air bubbles need to be removed and primed using the Flush Tether and Flush Buffer
- Key steps are to ensure air bubbles are not introduced into the ports
- The DNA library is mixed with sequencing buffer and loading beads
- The library is loaded to the SpotON sample port in a dropwise fashion.



<https://nanoporetech.com/>

FC- stored at RT unopened for 4 weeks or 2-8°C for 12 weeks and can be re-used once washed and stored for re-use

Principles of ONT sequencing



- The flow cells contain nanopores embedded in an electro-resistant membrane
- The nanopore only allows the passage of single-stranded DNA in a linear sequence using the motor protein
- When a molecule passes through the nanopore the current is disrupted to produce a 'squiggle'
- A unique pattern is produced by the different nucleotides passing through the nanopore
- Each nanopore has its own electrodes connected to a channel and sensor chip. The pattern is detected and interpreted to determine the nucleotide sequence

Data Analysis

- The signals are translated into DNA sequences using base calling algorithms that converts the raw current measurements into nucleotide sequences.
- This determines the DNA sequence in real time
- These electrical signals are recorded by the MinKNOW software and processed into reads
- Each read corresponds to a single strand of sequenced DNA
- Data is stored as FASTQ files
- The FASTQ files are then imported into the NanoTYPER software for genotyping analysis

NanoTYPER analysis

Genotyping | Result of 12 analyses | FAT77086_pass_barcode80_f25cde69_0_2023-04-14_11-18-44

Analysis name(s): FAT77086_pass_barcode80_f25cde69_0_2023-04-14_11-18-44, FAT77086_pass_barcode81_f25cde69_0_NA10005_2023-04-14_11-29-40, FAT77086_pass_barcode82_f25cde69_0_NA17290_2023-04-14_11-29-40

Sample Details, Browse Alignment, Setup Loci, Best Matches Only, Assignment State, Workflow State, Genotype Precision, Assignment, Comments, Send For Approval, Approve Result, Reject/Revoke Approval, Export Table, HMM Export, Turn LD on/off, Show LD details, Collapse/Expand

Displaying best matches only. Displaying 11 loci out of 11.

HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	HLA-DPB1	HLA-DRB3	HLA-DRB4	HLA-DRB5	HLA-DQA1	HLA-DPA1
FAT77086_pass_barcode80_f25cde69_0_2023-04-14_11-18-44										
02:01:01	44:02:01	05:01:01	04:01:01	03:01:01	04:01:01 04:01:63 1300:01 1321:01 1322:01 2 more allele(s)	No data present.	01:03:01:02N 01:03:01:13N	01:01:01 01:126	01:02:01	01:03:01
02:01:01	57:01:01	06:02:01	15:01:01 15:204	06:02:01	04:01:01 04:01:63 1300:01 1321:01 1322:01 2 more allele(s)	Allele not expected!			03:03:01	01:03:01
FAT77086_pass_barcode81_f25cde69_0_NA10005_2023-04-14_11-29-40										
02:01:01	15:03:01	02:10:01	04:01:01	03:01:01	04:01:01 04:01:63 1300:01 1321:01 1322:01 2 more allele(s)	No data present.	01:03:01	01:01:01 01:126	01:02:01	01:03:01
33:03:01	44:02:01	05:01:01	15:03:01 15:185	06:02:01	350:01	Allele not expected!	Allele not expected!	Allele not expected!	03:03:01	03:01:01#1

- Data are exported from NanoTYPER software as JSON, PDF, HML, CSV, XML, TXT format

NanoTYPER Quality Metrics

- The quality of results are assessed using various quality metrics
- Traffic light system, green, yellow, red
- This aids in accepting or rejecting your results

Genotype			Quality control			Data statistics		
Measure			HLA-A			HLA-B		
Overall ⓘ			PASSED			PASSED		
Primary QCs for Interpretation ⓘ								
Minimum coverage for key exon(s) ⓘ			● 572 ⓘ			● 939 ⓘ		
Key exon allele imbalance ⓘ			● 0.53 : 0.47 ⓘ			● 0.54 : 0.46 ⓘ		
Coverage ⓘ			● 987.32 ⓘ			● 1900.39 ⓘ		
Secondary QCs for Interpretation ⓘ								
Key exon maximum spot noise ratio ⓘ			● 16.79% ⓘ			● 24.38% ⓘ		
Other exon maximum spot noise ratio ⓘ			● 15.54% ⓘ			● 22.41% ⓘ		
PCR crossover artifact ratio ⓘ			● 11.94% ⓘ			● 13.12% ⓘ		
Minimum coverage for other exons ⓘ			● 403 ⓘ			● 232 ⓘ		
Other exon allele imbalance ⓘ			● 0.51 : 0.49 ⓘ			● 0.55 : 0.45 ⓘ		
Read length (N50) ⓘ			● 2098 ⓘ			● 1968 ⓘ		

Project

- Part of a wider project for DClinSci Higher Specialist Scientific training (HSST) looking at the utilisation of epitope prediction models in patients awaiting renal transplant.
- At the time of the project in 2022/2023 the laboratory was looking at options for HLA typing by NGS
- Retrospective project using data collected from patients transplanted between 2008 and 2015 who had received either a living or deceased donor kidney only transplant
- A total of 330 recipient and donor pairs were initially selected for the project
- These recipient and donor pairs were initially HLA typed using the local method at the time of transplant i.e. PCR-SSO/SSP
- Epitope prediction models require HLA typing data to be up to second field for accurate interpretation of model scores and mismatches at the amino acid level

Project Continued

- DNA from these recipient and donor pairs were originally stored between -40 to -80°C and were identified for TGS typing (660 samples)
- TGS typing was performed using NanoTYPE™ HLA amplification kits (NT24/11v2) as per manufactures guidelines
- 638 samples were successfully typed using NanoTYPE™ kits
- 11 recipient and donor pairs were excluded from the project due to poor DNA quality or limited DNA
- Data was collected using MinKNOW software version 22.05.5 and FASTQ files were imported and analysed using NanoTYPER® V.2.0.0 RUO

Results

Quality Metric	A* (%)	B (%)	C* (%)	DRB1/3/4/5 (%)	DQA1 (%)	DQB1 (%)	DPA1 (%)	DPB1 (%)	Total (%)
Passed	85.22	94.64	93.49	95.15	91.09	92.14	96.87	94.83	92.93
Inspect	1.39	0.64	0.88	0.65	2.22	1.32	0.08	0.96	1.02
Failed	10.35	2.74	3.65	0.99	4.26	4.57	1.07	1.83	3.60
No data	3.04	1.98	1.98	3.22	2.44	1.98	1.98	2.28	2.30
Concordance*	99.6	99.85	99.07	98.3	99.85	98.92	100	97.82	99.18
Discrepancy*	0.4	0.15	0.93	1.7	0.15	1.08	0	2.18	0.82

* Does not include samples with no data

Known Ambiguities and Allele Dropouts

- The observation of allele dropout is a known limitation for long range PCR
- Known product limitations can be found on the <https://omixon-download>
- Some of the commonly identified allele dropouts included:
 - DQB1*03:01,
 - DQB1*03:02,
 - DQB1*04:01,
 - DRB1*04
 - DRB1*07:01
- Samples that had allele dropouts were repeated and found to be in accordance with the original result.
- A further known limitation is around typing for HLA-DRB4*03:01N as this gene is not amplified due to the deletion of the forward primer.
- There were only four samples affected by this within the project.

Discrepancy at HLA-B

- A living donor was originally typed as HLA-B*07 and B*42 by PCR-SSO
- The NGS result was HLA-B*07:01 and B*08:01
- This was further investigated by repeating the Omixon TGS and also re-typing using the local NGS method
- The local NGS and Omixon TGS methods both were concordant with each other and confirmed as HLA-B*07:01 and HLA-B*08:01
- The donor HLA type was updated in the laboratory LIMS and the impact of the change assessed
- This was a living donor transplant previously identified as being matched for HLA-B*07 and a mismatch for HLA-B*42. The patient's antibody profile was reassessed for the presence of antibody to the newly identified HLA-B*08 mismatch
- No antibody to either HLA-B8 or B42 had been identified, so there was no clinical impact to the patient

Discrepancy at HLA-C

- Omixon TGS typing kits determined one patient as being HLA-C*06:01, *16:01
- The previous typing by PCR-SSO had defined the patient as being HLA-C*06:01 homozygous
- The sample was repeated using Omixon TGS kits and local NGS method, which confirmed the presence of C*16:01
- This patient had received a deceased donor transplant that, based on historic typing, had been assigned as having a mismatch for HLA-C*16:01, but matched for HLA-C*06:01
- This error in the initial typing of the patient had no clinical impact as they are now defined as matched at HLA-C*
- The patient HLA type was corrected within the laboratory LIMS system

Initial limitations

- The initial limitations of the assay included:
 - **User inexperience:** This was overcome with further training. Mostly due to priming and loading the sample onto the flow cell
 - **Incorrect storage of barcoding kits:** This was overcome by correct storage of reagents in a temperature-controlled fridge
 - **Incorrect storage of flow cells:** DO NOT FREEZE. Pores will become inactive. Correct washing important to maintain pores
 - **Short expiry dates for flow cell:** This wouldn't be an issue if used routinely due to higher turnover of routine work
 - **Stable internet connection:** This could be prevented by ensuring software is on a networked computer, so the internet connection remains stable
 - **DNA quality:** The assay requires DNA to be extracted and stored correctly

Benefits

- The MinION takes up very little bench space and is portable
- A low initial start up cost for equipment
- Ability to re-use flow cells for multiple runs as long as washed correctly and stored correctly
- Quick turn-around - A typical batch of 24 samples typically took 24 hours from sample and library preparation to sequencing and analysis
- Pushed the limits, 48 samples can be prepped and sequenced over a weekend with minimal repeats
- The MinION can run for 72 hours but is not recommended and decreases the MinION life
- Easy workflow with simple processes and safe places to stop
- Analysis was easy with quality metrics guiding when samples needed repeating
- The library can be re-run if there is a low output with FASTQ files combined for analysis

Summary

- HLA typing by TGS can be performed using NanoTYPE kits in combination with ONT sequencer
- There is very good concordance with previous results
- Little start up costs involved and minimal lab space required
- Workflow is simple with safe stopping points
- Can HLA type from 1-48 samples
- Limitations were mostly due to lab environment (internet) and user inexperience with DNA methods
- 638 samples were successfully HLA typed using the NanoTYPE kits
- **The future:** We are currently in the process of implementing this technique routinely as alternative for current NGS method, urgent single sample testing, disease association, repeats and allele dropouts from the current method

Acknowledgements

Clinical Transplantation Laboratory

- Olivia Shaw
- Corinna Freeman
- Sarah Blears

Omixon

- Peter Romada
- Mazen Mabrok
- Nina Lauterbach
- Omixon support

Thank you for all the support and early adopter kits

Scientific Learning and Development Fund (Synnovis)

Oxford Nanopore Technology Customer Services

Transplant, Renal and Urology Research Project Board at Guy's Hospital

University of Manchester

Werfen