Pooling HLA, Chimerism, and cfDNA NGS Libraries for Sequencing on Illumina Instruments

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AIM

The purpose of this study is to evaluate the feasibility of combining HLA and Chimerism or HLA and cfDNA NGS libraries before sequencing to minimize turnaround times and cost per sample. Key differences between NGS libraries were identified and adjustments prior to and after sequencing were made. Combined libraries were sequenced on the Illumina MiSeq, MiniSeq and iSeq instruments.

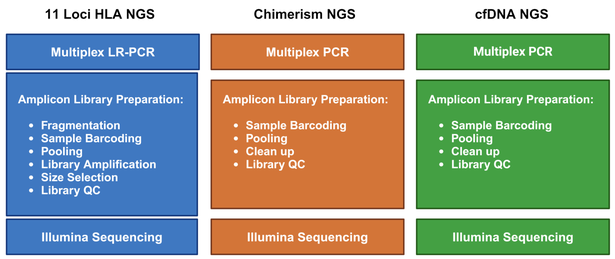


Figure 1. NGS workflows for HLA, Chimerism, and cfDNA.

NGS library preparation steps for HLA, Chimerism and cfDNA are shown in **Figure 1**. Instructions from the manufacturer were followed up to the point of final library QC with fluorometric method. Differences in fragment length, total reads requirements, read and index length, and de-multiplexing strategy were considered in this study (**Figure 2**).

	11Loci HLA NGS	Chimerism NGS	cfDNA NGS
Average Fragment Length	825bp	213bp	217bp
Number of Reads Required	*166,000	**32,000 S ***320,000 M	**68,000 S ***340,000 M
Sequencing Mode	2 x 151	2 x 76	2 x 76
Index Length	8bp	10bp	10bp
De-Multiplexing Approach	Single Index	Dual Index	Dual Index

*Theoretical minimum number of reads required for typing. * Desired number of reads for Screening and **Monitoring samples used for Chimerism or dd-cfDNA analysis.

Figure 2. Key considerations for dual NGS assay sequencing.

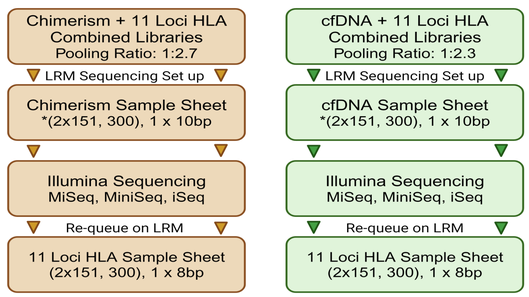
METHODS

Six control samples with known chimeric status ranging from 0.1% to 50% of recipient DNA and 0.5% to 30% of dd-cfDNA were used for NGS library prep. Each pool contained donor and recipient samples for detection of informative markers. The 11 Loci HLA NGS libraries contained eight to twenty-nine samples in the final pool. NGS final library concentrations for chimerism and cfDNA were slightly adjusted for better clustering on Illumina instruments (**Table 1**).

Table 1. Dual Assay Sequencing Library Concentrations

NGS Library	MiSeq	MiniSeq	iSeq
11 Loci HLA	2.1 ng/uL	0.53 ng/uL	0.056 ng/uL
Chimerism	0.22 ng/uL	0.05 ng/uL	0.006 ng/uL
cfDNA	0.27 ng/uL	0.06 ng/uL	0.007 ng/uL

The pooling volumes for Chimerism and 11 Loci HLA or cfDNA and 11 Loci HLA libraries were determined with a Sequence Coverage Calculator Tool[v]. The pooling ratios were approximately 1:2.7 and 1:2.3. After NGS libraries were combined, libraries were prepared for sequencing as per the 11 Loci HLA NGS protocol. The chimerism or cfDNA sample sheet was uploaded to the Local Run Manager prior to sequencing. After sequencing was completed, the 11 Loci HLA sample sheet was uploaded and re-queued to recover indexed reads. Both de-multiplexing events utilized a single index. MiSeq v2 Micro kits, MiniSeq Mid Output kits, and iSeq 100 v2 kits were used for sequencing dual NGS libraries.



***Note:** Sequencing mode on MiniSeq runs was 2x149 due to reagent limitation in the number of sequencing cycles.*

Figure 3. Modified workflow for sequencing dual NGS libraries.

RESULTS

Table 2. HLA typing concordance for dual NGS Illumina runs.

Dual Library	Platform	3-F Concordance
HLA + Chimerism	MiSeq (n=16)	100%
HLA + Chimerism	MiniSeq (n=37)	100%
HLA + Chimerism	iSeq (n=16)	100%
HLA + cfDNA	MiSeq (n=16)	100%
HLA + cfDNA	MiniSeq (n=37)	100%
HLA + cfDNA	iSeq (n=16)	100%

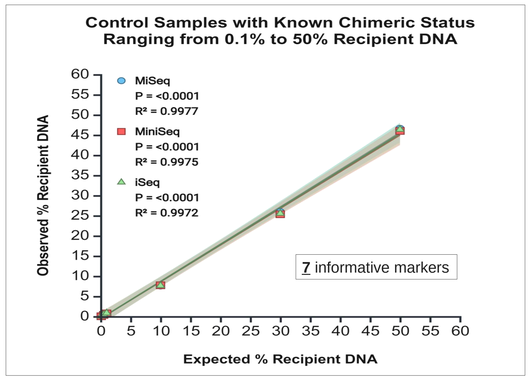


Figure 4. Theoretical and observed chimerism on Illumina runs.

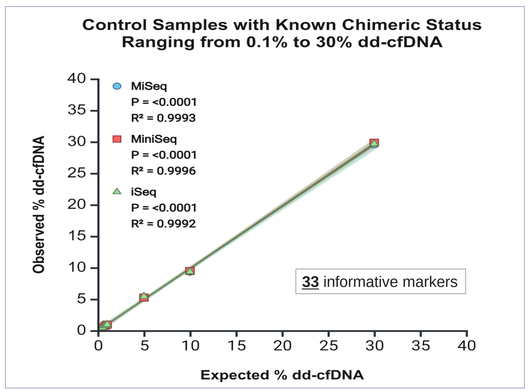


Figure 5. Theoretical and observed dd-cfDNA on Illumina runs.

CONCLUSIONS

- Feasibility experiments suggest that combining two NGS library types in one sequencing run is a viable strategy to minimize turnaround time and reduce cost per sample.
- Adjustments in library concentration, pooling ratio, sequencing and demultiplexing strategy were implemented to achieve accurate HLA typing, robust identification of informative markers, and consistent analysis of chimerism and/or dd-cfDNA.
- Although in some cases the recommended read depth was not achieved, concordant HLA typing, chimerism, and/or dd-cfDNA results were obtained for all control samples in this study.
- Further optimizations in pooling ratios for chimerism and 11 Loci or cfDNA and 11 Loci HLA are required to ensure optimal read depth for all samples in sequencing runs with combined NGS library types.

[v]Sequence Coverage Calculator Tool:
https://calculator-for-chimerism.devyser.com/

TRADEMARKS/LICENSING