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Technical Note

Pooling HLA NGS and Chimerism NGS or HLA NGS and cfDNA NGS libraries for simultaneous sequencing on Illumina instruments

Introduction

Within the clinical environment, turnaround time (TAT) and cost per sample are critical factors for workflow and Next Generation Sequencing (NGS) technology integration. Optimization efforts on NGS workflows and sequencing can effectively minimize both the TAT and cost per sample. One such strategy involves the pooling of different NGS library types before sequencing. This approach maximizes use of the Illumina flow-cell, leading to a reduction in cost per sample. Additionally, it eliminates the need to conduct separate sequencing runs for different assays.

In a typical laboratory setting, HLA sequencing, chimerism analysis, and/or dd-cfDNA analysis are commonly employed assays. With careful considerations and avoiding index sequence overlap, these assays can be sequenced simultaneously. However, this approach requires some adjustments in the library dilution, pooling, and sequencing to account for differences in library size, read depth requirements, and demultiplexing analysis. Under standard conditions, chimerism and cfDNA NGS libraries are sequenced using 2x76 with 2x10bp indexes, while libraries from 11 Loci HLA NGS assays are sequenced with 2x151 and 1x8bp or 2x8bp indexes. Differences in library size make it challenging to accurately predict clustering efficiency on Illumina flow-cells, leading to potential distortions in read distribution and coverage between NGS libraries.

In this technical note, we introduce various pooling ratios between different NGS assays and discuss library dilution methods employed to achieve adequate distribution of reads and coverage for simultaneous sequencing of two NGS library types.

Table 1. Key considerations, single assay sequencing

	11 Loci HLA NGS	Chimerism NGS Screening Protocol	Chimerism NGS Monitoring Protocol	cfDNA NGS Screening Protocol	cfDNA NGS Monitoring Protocol
Average library size (bp)	600-825	213		217	
No. of reads required per sample (theoretical)	166,000	32,000	320,000	68,000	340,000
Sequencing Mode	2x151, 1x8bp or 2x151, 2x8bp	2x76, 2x10bp			

Table 2. Single assay library concentration

	MiSeq	MiniSeq	iSeq 100
11 Loci HLA NGS	Assay-dependent	Assay-dependent	Assay-dependent
Chimerism NGS	0.3 ng/µL	0.21 ng/µL	0.01 ng/µL
cfDNA NGS	0.37 ng/µL	0.23 ng/µL	0.01 ng/µL

Methods: Library generation

Chimerism NGS

Two screening samples and six monitoring samples with the following chimerism status were prepared and diluted to 6 ng/µL:

- 0.1% recipient
- 0.5% recipient
- 1% recipient
- 10% recipient
- 30% recipient
- 50% recipient

The eight samples underwent amplification using chimerism assay reagents following manufacturer instructions, up to the point of library dilution prior to denaturation.

CfDNA NGS

Two screening samples and six monitoring samples with the following dd-cfDNA status were prepared and diluted to 0.6 ng/µL:

- 0.5% dd-cfDNA
- 0.8% dd-cfDNA
- 1% dd-cfDNA
- 5% dd-cfDNA
- 10% dd-cfDNA
- 30% dd-cfDNA

The eight samples underwent amplification using cfDNA NGS reagents following manufacturing instructions, up to the point of library dilution prior to denaturation.

11 Loci HLA NGS

Eight to twenty-nine QC DNA samples were diluted to 15-30 ng/µL and then subjected to 11 Loci multiplex PCR amplification and library preparation using HLA NGS reagents.

Pooling and sequencing strategies

Adjustments to the library concentration of the chimerism NGS and the cfDNA NGS were required prior to pooling (refer to Table 3 for the concentrations utilized for pooling). The 11 Loci HLA NGS libraries were diluted as instructed in the manufacturer protocol. For each Illumina instrument, the pooling ratio between chimerism NGS or cfDNA NGS with the 11 Loci HLA NGS library was 1:2.7 and 1:2.3 respectively.

Table 3. Concentrations used for pooling NGS libraries

	MiSeq	MiniSeq	iSeq 100
11 Loci HLA NGS	Assay-dependent	Assay-dependent	Assay-dependent
Chimerism NGS	0.22 ng/µL	0.05 ng/µL	0.006 ng/µL
cfDNA NGS	0.27 ng/µL	0.06 ng/µL	0.007 ng/µL

A sequence coverage calculator was employed to determine the pooling volume of NGS libraries, as depicted in Figure 1. In the sequence coverage calculator, the 11 Loci HLA NGS library was designated as “Other,” and the “Number of reads/read pairs” input was 166,000 multiplied by the number of samples.

The standard dilution and denaturation method for the 11 Loci HLA NGS were used for sequencing the combined pool on the different Illumina instruments.

The following number of samples were sequenced together on the different Illumina platforms:

Table 4. 11 Loci HLA NGS and Chimerism NGS Batch Sizes

	11 Loci HLA NGS	Chimerism NGS Screening Protocol	Chimerism NGS Monitoring Protocol
MiSeq-Micro Flow cell	8	2	6
MiniSeq-Mid Flow Cell	29	2	6
iSeq -reagent v2	8	2	6

Table 5. 11 Loci HLA and cfDNA Batch Sizes

	11 Loci HLA NGS	cfDNA NGS Screening Protocol	cfDNA NGS Monitoring Protocol
MiSeq-Micro Flow cell	8	2	6
MiniSeq-Mid Flow Cell	29	2	6
iSeq -reagent v2	8	2	6

A notable distinction between the chimerism NGS or cfDNA NGS and the 11 Loci HLA NGS was the sequencing configuration used in the sample sheet. The former was set as 2 x 151 and 1x10bp or 2x10bp depending on the demultiplexing strategy of the latter (i.e. single or dual index). Upon completion of the sequencing run, fastq files for the chimerism NGS or cfDNA NGS libraries were obtained, and a secondary analysis was initiated by re-queueing with the sample sheet including index sequences for the 11 Loci HLA NGS library. A schematic workflow for the combined NGS assay sequencing approach is shown in Figure 2.

Date:	
Sequencing system:	Illumina MiSeq
Sequencing kit/flow cell:	MiSeq Reagent Kit v2 Micro (300 cycles)
Remaining capacity:	6.1%
PhiX control:	1% (35,640 reads)
Capacity comment:	

Name amplicon pool	kit / variant	Number of samples	Minimal coverage per variant allele	Minimal coverage per amplicon	Mean coverage per amplicon	Variant Allele Frequency (VAF)	Number of reads/read pairs	Used kit capacity	% of run	Volume of amplicon pool [µl]
Chimerism / Screening NGS Library		2	500	1000	1333	N/A	64,000	1.8%	1.9%	2.0
Chimerism / Monitoring NGS Library		6	5000	10000	13333	N/A	1,920,000	53.9%	58.0%	60.3
11 Loci HLA NGS Library		N/A	N/A	N/A	N/A	N/A	1,328,000	37.3%	40.1%	41.7

Total Number of Reads Required for Each Library   Pooling Volumes for Each Library

Figure 1. Example of the Sequence Coverage Calculator report for the 11 Loci HLA NGS and Chimerism NGS libraries sequenced on the Illumina-MiSeq instrument.

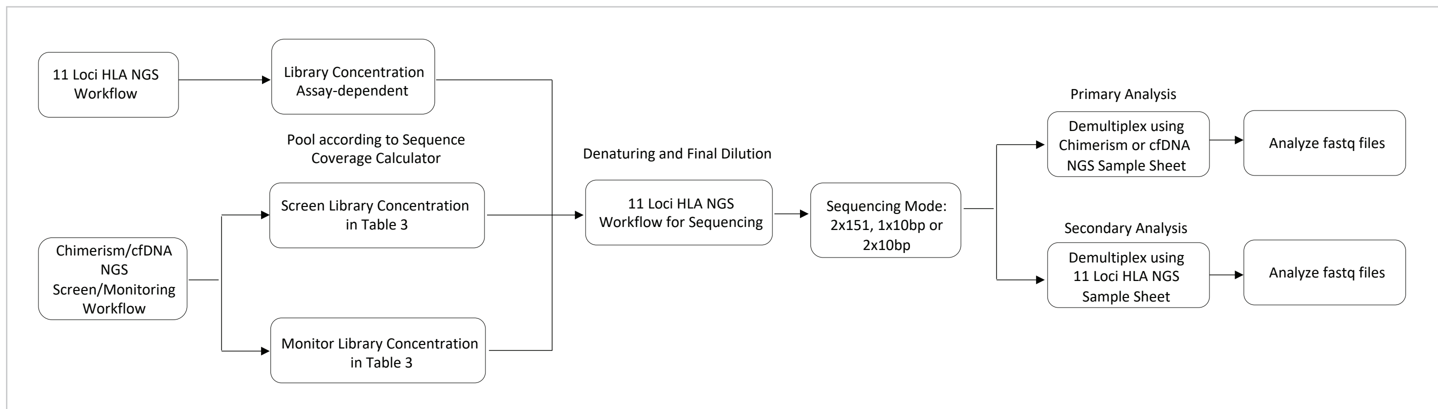

Figure 2. Experimental workflow for sequencing of 11 Loci HLA with chimerism or 11 Loci HLA with cfDNA NGS libraries.

Results

Distribution of reads

The distribution of reads between the assays differed slightly from the theoretical value given in the Sequence Coverage Calculator (Figure 3).

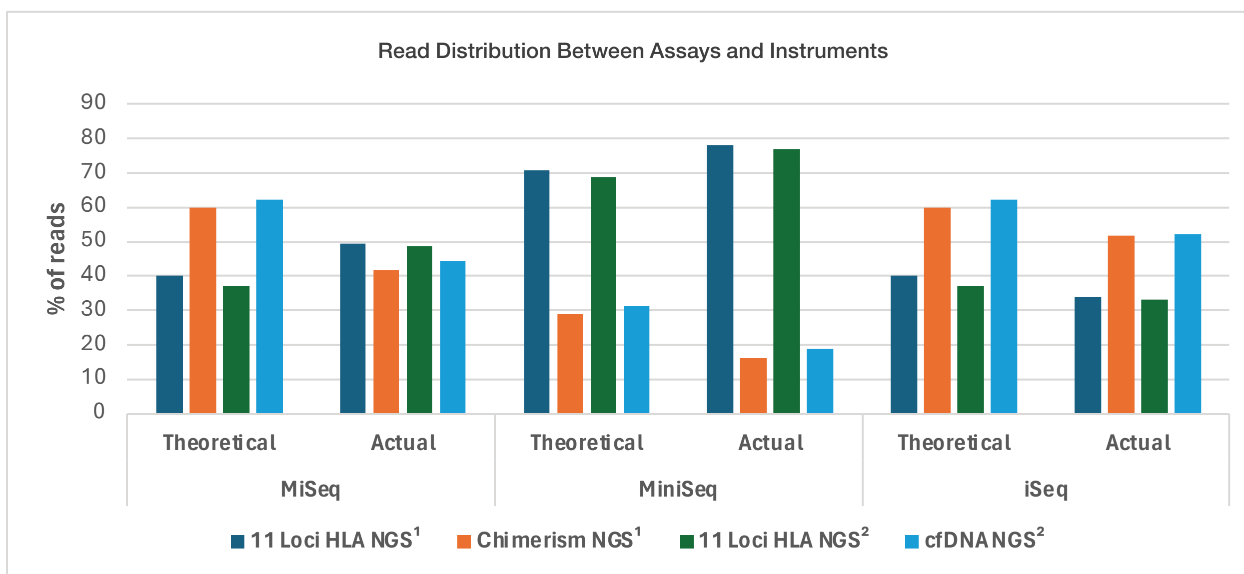

Figure 3. Theoretical versus actual distribution of reads between 11 Loci HLA NGS and chimerism NGS or 11 Loci HLA NGS and cfDNA NGS assays on different Illumina platforms.

All 11 Loci HLA NGS samples surpassed QC read depth requirements of 200x for all Illumina platforms. However, not all chimerism NGS samples met the required read depth for the chimerism screening and monitoring protocol. The lowest number of reads was 26,069 and 287,243 reads for a screening and a monitoring sample in a MiSeq run, respectively.

A similar pattern was observed in the cfDNA NGS samples, where the lowest number of reads for a screening sample was 59,696 and 285,094 in a MiniSeq run. All screening and monitoring samples achieved the required read-depth in the iSeq 100 sequencing runs.

Chimerism NGS

All screening samples revealed the presence of an identical number of informative markers (i.e. 7 markers total) across sequencing instruments. Each marker was accurately identified as either homozygote or heterozygote in the screening samples even though some samples did not achieve the recommended number of reads.

The monitoring samples showed consistent measurements of recipient DNA across Illumina instruments, with all data sets exhibiting an R2 between 0.996 and 0.998, as illustrated in Figure 4.

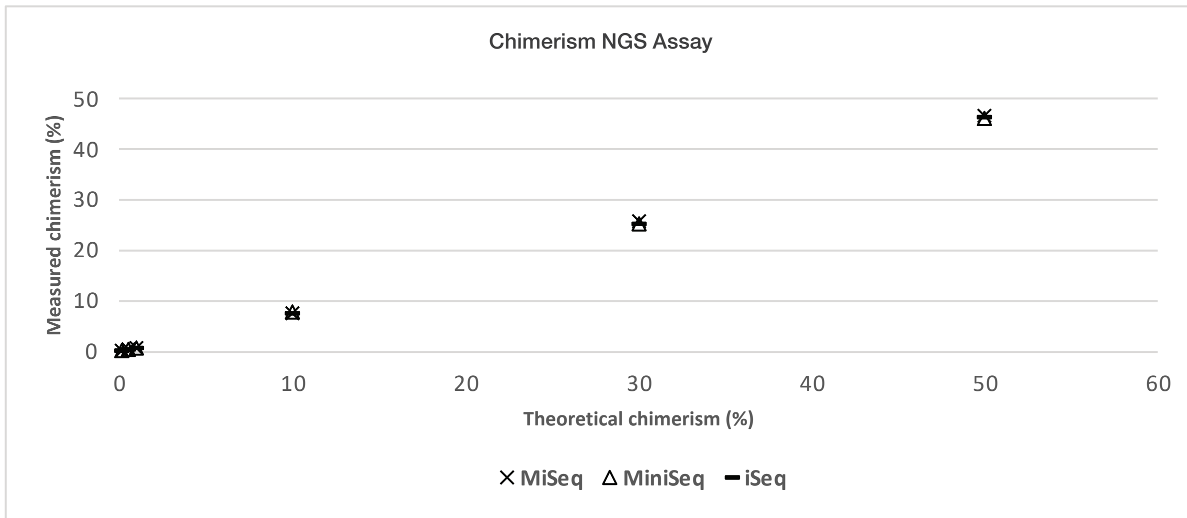

Figure 4. Theoretical and measured percentage of chimerism across Illumina instruments.

cfDNA NGS

All screening samples revealed the presence of an identical number of informative markers (i.e. 33 markers total). Each marker was accurately identified as either homozygote or heterozygote in the screening samples despite some samples not achieving the required number of reads.

The observed percentage of dd-cfDNA was consistent across Illumina instruments, with all datasets exhibiting an R2 > 0.999, as illustrated in Figure 5.

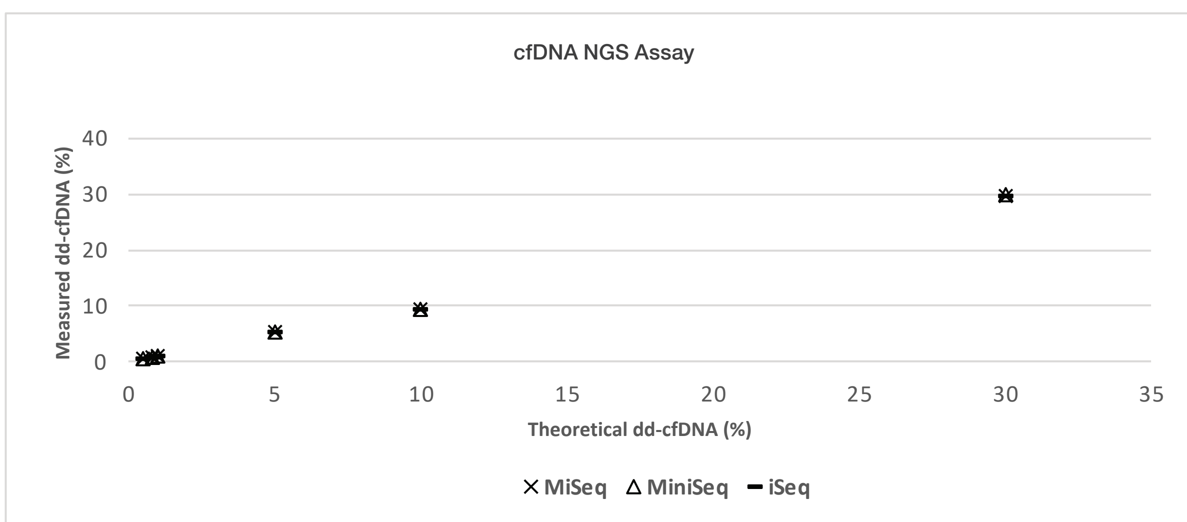

Figure 5. Theoretical and measured %dd-cfDNA across Illumina instruments.

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11 Loci HLA NGS

HLA typing concordance for all Quality Control samples was 100% at 3-field across three different Illumina instruments. The average read depth in key exons for Class I and Class II HLA genes was over 200x and the average allele balance was over 0.3 for all allele combinations tested. Normal read distribution (CV < 30%) was observed for all HLA libraries mixed with chimerism NGS or cfDNA NGS libraries.

Conclusion

In conclusion, the presented experiments demonstrate the feasibility of sequencing 11 Loci HLA NGS, chimerism NGS, and cfDNA NGS simultaneously on Illumina instruments. The key objectives of minimizing turnaround time (TAT) and reducing cost per sample were addressed through the strategic pooling of NGS libraries before sequencing. Despite the inherent challenges posed by variations in library lengths and number of sequencing cycles, the experiments successfully employed dilution methods to achieve desired ratios between different assays.

The pooling and sequencing strategies, as outlined in the methods section, allowed for the simultaneous sequencing of 11 Loci HLA NGS, chimerism NGS, and cfDNA NGS on the MiSeq, MiniSeq, and iSeq instruments. Notably, adjustments in the loading concentrations were implemented to accommodate the distinct dilution strategies used post-denaturation for 11 Loci HLA NGS and chimerism or cfDNA NGS libraries. A sequence coverage calculator facilitated the calculation of volume ratios used for pooling, ensuring optimal utilization of Illumina flow-cells.

Furthermore, the chimerism NGS and cfDNA NGS demonstrated accurate identification of informative markers even when the recommended read-depth was not achieved in some cases. High resolution HLA typing with 100% concordance at 3-Field was achieved with the 11 Loci HLA NGS assays evaluated in this study.

Disclaimer

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MKT-AD-OLI-4760 | AUG 2024 | REV 00