Simplified Technique-Sensitive Washing Steps for a Robust Hybrid Capture Target Enrichment NGS Assay

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Introduction

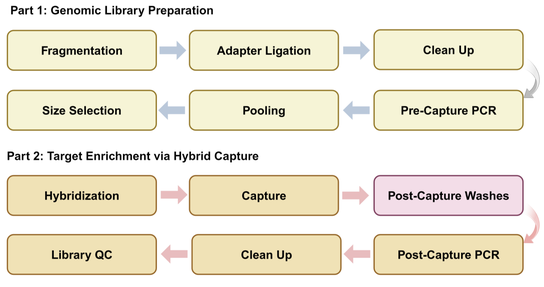
Hybrid capture target enrichment for HLA typing by Next generation sequencing (NGS) overcomes some of the limitations of long-range PCR enrichment, e.g., amplification failure due to novel SNP in the primer binding site, poor sample quality, amplification bias, etc. The washing step in the hybrid capture assays is technique sensitive and is critical to the quality of the data the assay produces. In this study, our aim was to improve the robustness of the hybrid capture assay by simplifying the washing steps and making it more tolerant towards technique variations between users.

Materials and Methods

Sample Preparation and Test Method(s)

In this study, 96 well-characterized samples were used to create a genomic library. The same genomic library was carried through the target enrichment workflow in quadruplicate and only varied at the post-capture washes step of the target enrichment procedure, shown in Figure 1.

Figure 1. Overview of the Hybrid Capture NGS Assay



The study focused on investigating the removal or simplification of the most sensitive parts of the hybrid capture target enrichment washing steps: (i) pre-heating the wash buffer at 65˚ C prior to addition to the bead-capture mix and (ii) heating the bead-capture mix with wash buffer at 65˚ C during wash incubations and (iii) reducing the incubation time of the heated wash steps. Testing conditions A-D are outlined in Table 1.

Table 1. Variations Tested During Optimization of Critical Post-Capture Washing Steps

Condition	Pre-Heated Buffers	Wash Buffer 1		Wash Buffer 2	
		# of Washes	Time	# of Washes	Time
Control - A	Y	2 Heated	5 min	2 Heated	5 min
B	N	2 Heat Incubated	3 min	2 Heat Incubated	3 min
C	N	2 Room Temp	N/A	2 Heat Incubated	3 min
D	N	2 Room Temp	N/A	2 Room Temp	N/A

Each of the final enriched 96-plex libraries were sequenced on the Illumina MiSeq with Standard Flow Cell V2 (300 cycle) Kit.

Data Analysis

The quality of the on-target rate, read coverage, and HLA typing concordance generated with the genotyping software from the experimental washing conditions were compared against a standard washing protocol (control).

Results

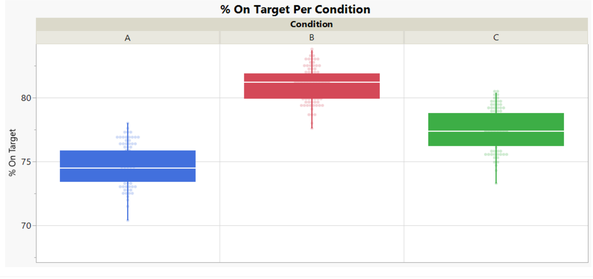
Results from Simplified Washes

Table 2. Final Library Yield Experimental wash conditions B and C gave final library yield comparable to condition A (control). Condition D was abnormally high and outside of expected range, and the library was not sequenced.

Condition	Final Library Yield (ng/µL)
Control – A	9.71
B	7.17
C	9.56
D	>90

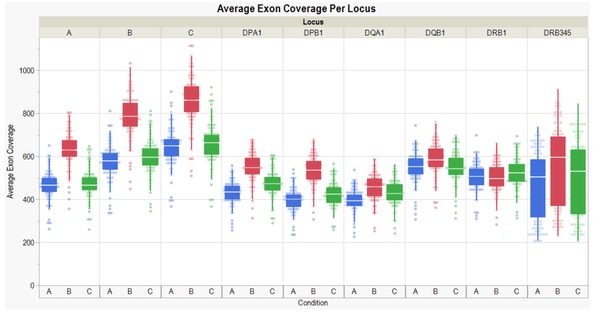
It was observed that elimination of heat from the all washing incubation steps for condition D produced a high library yield. Since the library yield was outside of the acceptable library yield range and failed to enrich for the targets, the library was not sequenced.

Figure 2. % On Target Per Sample for Each Wash Condition Experimental conditions B and C generated a higher on target rate than condition A (control) for all 96 samples.



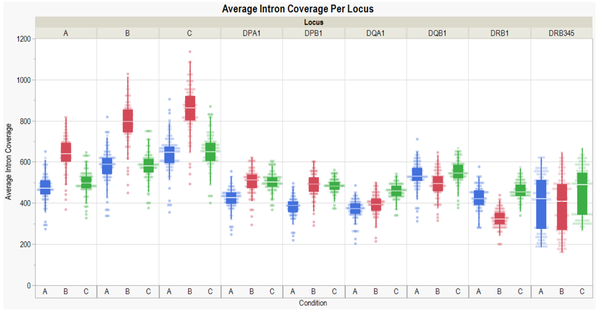
Experimental wash conditions B and C generated higher on-target rates, despite the omission of pre-heating steps for Wash Buffer 1 and the reduced heated incubation times for Wash Buffer 2. Condition B, which had all heat incubated washes, had the highest on-target value, suggesting that this was the most stringent of the tested conditions.

Figure 3. Average Exon Coverage Per Locus Average exon coverage for A, B, C, DPA1, DPB1, DQA1, and DRB345 loci were improved with experimental wash conditions. In these loci, experimental conditions B and C outperformed control condition A (control), with condition B being the highest. The coverage of DQB1 and DRB1 loci was not significantly impacted by the wash conditions and remained comparable to condition A (control).



Average exon coverage in all loci for experimental conditions B and C were greater than or equal to that of the condition A (control).

Figure 4. Average Intron Coverage Per Locus Average intron coverage plots for A, B, C, DPA1, DPB1, DQA1 and DRB345 loci were improved with experimental wash conditions. The average intron coverage for condition C in DQB1 and DRB1 loci was comparable to the control, however condition B showed decreased coverage for DQB1 and DRB1 loci.



Average intron coverage for A, B, C, DPA1, DPB1, and DQA1 were higher for experimental wash conditions B and C. Average intron coverage for DQB1, DRB1 and DRB345 loci for experimental wash condition B were lower coverage than the condition A (control), despite having a higher on-target. The average intron coverage for DRB1 was higher coverage in experimental condition C than condition A (control), while still maintaining coverage in DQB1 and DRB345 loci. This data suggests that having two room temp Wash Buffer 1 washes and two heat incubated Wash Buffer washes increases on-target rate without negatively impacting coverage.

Table 3. HLA Concordance per Locus HLA concordance for condition A (control), B and C were all 100% concordant at the 3rd field.

Targets	A	B	C
	# of Concordant Alleles	# of Concordant Alleles	# of Concordant Alleles
HLA-A	192/192	192/192	192/192
HLA-B	192/192	192/192	192/192
HLA-C	192/192	192/192	192/192
HLA-DRB1	192/192	192/192	192/192
HLA-DRB345	159/159	159/159	159/159
HLA-DPA1	192/192	192/192	192/192
HLA-DPB1	192/192	192/192	192/192
HLA-DQA1	192/192	192/192	192/192
HLA-DQB1	192/192	192/192	192/192
% 3rd Field Concordance	**100%** (1,695/1,695)	**100%** (1,695/1,695)	**100%** (1,695/1,695)

The HLA typing concordance for the experimental washes was comparable to control, with ≥99.9% at 3rd field.

Conclusions

This study demonstrated that critical washing steps in the NGS hybrid capture assay can be simplified by eliminating, the need to pre-heated wash buffers, the incubation above 25˚ C for the first wash step, and reducing the time of the second heated wash steps. These changes to the wash procedure increased on-target and raised coverage in DRB1 and DRB345 loci.

These optimizations significantly minimized variability in the on-target results from hybrid capture target enrichment and increased the robustness of the assay, leading to a significantly improved and simplified protocol that is more reproducible and easier to carry out.

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