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Evaluation of long-read capture enrichment for Nanopore sequencing of *RHD* and *RHCE* to resolve complex haplotypes

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Background: Determining the complete allelic composition of the highly homologous *RHD* and *RHCE* genes is essential for transfusion medicine, yet this remains challenging due to their complex genomic organisation. Despite their ability of phasing genetic variants, existing targeted approaches for Nanopore sequencing – such as overlapping long-range PCR and adaptive sampling – encounter limitations, including allele drop-out, uneven coverage, and modest enrichment efficiencies. To overcome these challenges, a capture-enrichment approach was evaluated for targeting *RHD* and *RHCE*, complemented by the *RHAG* gene, which can modulate RhD/RhCE expression.

Methods: Custom capture probes were designed to span the genomic interval from *RHD* (~57 kb) to *RHCE* (~59 kb), including 75 kb of flanking regions on either side. The probe panel was extended to also target *RHAG* with 10 kb of additional flanking sequences. For this proof-of-concept study, eight samples were pooled and sequenced on a single MinION flow cell. Data analysis employed both *de novo* and reference-based variant calling pipelines to assess coverage uniformity, variant phasing accuracy, and the resolution of hybrid alleles.

Results: The enrichment approach produced evenly distributed coverage over the targeted region with an average depth of ~1600× and an N50 of 3.7 kb. Between 37.8% and 41.7% of the sequencing reads mapped to the regions of interest. Variant phasing within and between *RHD* and *RHCE* was successful in non-hybrid samples, with all exonic single nucleotide variants (SNVs) aligning with previous findings. In a reference-based setting, hybrid allele breakpoints could be defined with high resolution—particularly when a *RHCE*02* reference sequence was used to reduce mismappings in RhC-positive samples. However, initial attempts at *de novo* assembling hybrid alleles were impeded by the moderate read length distribution.

Conclusions: This study demonstrates that long-read capture enrichment combined with Nanopore sequencing can effectively target the complex *RHD*/*RHCE* locus, in combination with other loci such as *RHAG*. The method offers significant advantages over amplicon-based techniques by delivering more uniform coverage and improved breakpoint determination, and it is more cost-effective than adaptive sampling. Protocol optimizations to achieve longer reads (targeting an N50 of up to 7 kb) could enhance mapping and phasing, underscoring the promise of this approach for full-length sequencing of complex blood group genes.