# A Nanopore Sequencing Assay for Comprehensive Molecular Characterization of Hemoglobinopathy-Associated Variants

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## SUMMARY


- **Hemoglobinopathies are among the most commonly inherited genetic disorders, each with high carrier rates that often require distinct, specialized genotyping methods.**
- **An expanded amplification-based long-read nanopore sequencing prototype was developed to increase genomic coverage across α- and β-globin clusters, including regulatory regions and clinically relevant modifier loci, within a unified workflow.**
- **Enhanced target design enables a scalable, and flexible framework for detection and interpretation of variant types and genomic regions not fully covered by conventional globin-focused assays.**


## INTRODUCTION

Hemoglobinopathies represent one of the most common groups of monogenic disorders worldwide, caused by a wide spectrum of pathogenic variants in the α- and β-globin cluster genes (e.g., *HBA1/2* and *HBB*) resulting in α- and β-thalassemia. When using only short-read sequencing technologies, high intra-cluster sequence homology amongst α- and β-cluster genes and the presence of large deletions, duplications, and complex rearrangements complicate accurate genotyping. Therefore, multiple specialized and complementary molecular methods are often required for comprehensive molecular diagnosis.

The currently available amplification-based long-read nanopore sequencing assay, AmplideX® Nanopore Carrier Plus Kit C (RUO), was created with the capability to screen for common variant types in the *HBA1/2* and *HBB* genes. The work presented here expands coverage to include all known disease-causing variants in the α- and β-globin cluster genes. It additionally captures key regulatory elements, relevant modifiers, and other assay improvements, providing a more comprehensive molecular characterization of thalassemia and related disorders within a unified workflow.

## METHODS

Genomic DNA extracted from 70 whole blood clinical samples and 30 reference cell lines (Coriell Cell Repository) was amplified using AmplideX® PCR chemistry, followed by barcoding, pooling, and library preparation with the ONT Ligation Sequencing Kit (SQK-LSK114). Sequencing was performed on R10.4.1 flow cells using a GridION® or MinION® Mk1D platform. Custom-built software was developed to automate sequencing setup/execution, monitor performance metrics, perform secondary analysis, and generate genotype reports. An initial training set was used to develop algorithms for detecting large deletions and duplications, while an independent panel of challenge samples containing additional variant types was used for performance evaluation. The AmplideX® Nanopore Carrier Plus Kit (RUO)[1] and GAP-PCR were used as comparator methods.


**Figure 1. AmplideX Nanopore Carrier Plus Kit Panel Design and Workflow.** Workflow consisted of PCR enrichment, sequencing, and automated analysis software. Basecalled sequencing data was analyzed with custom-built, automated software to report multiple variant classes across genes.

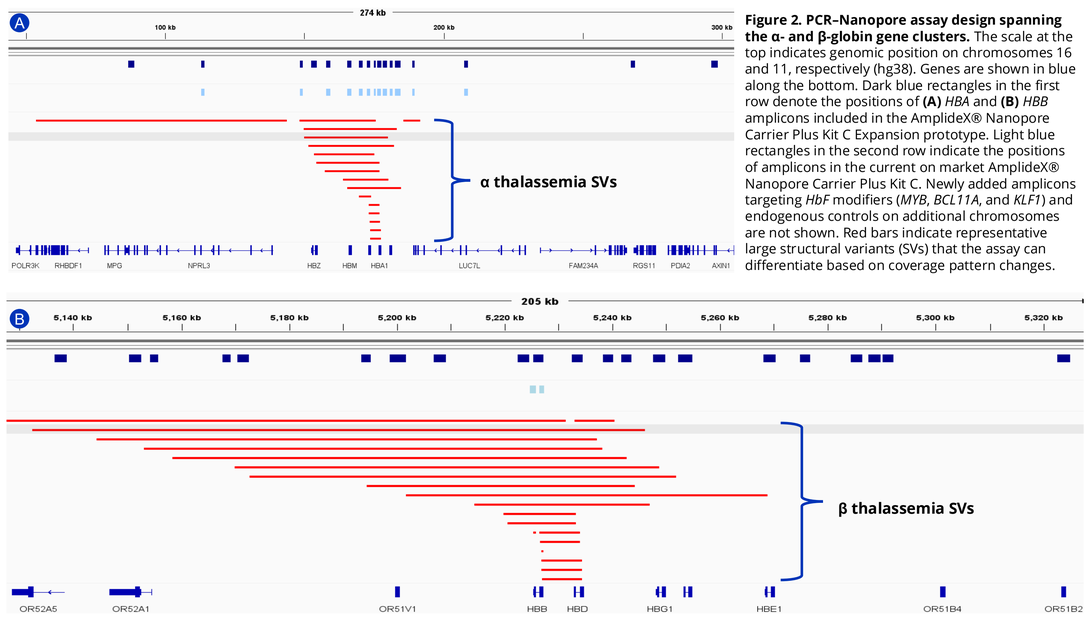


**Figure 2. PCR–Nanopore assay design spanning the α- and β-globin gene clusters.** The scale at the top indicates genomic position on chromosomes 16 and 11, respectively (hg38). Genes are shown in blue along the bottom. Dark blue rectangles in the first row denote the positions of **(A)** *HBA* and **(B)** *HBB* amplicons included in the AmplideX® Nanopore Carrier Plus Kit C Expansion prototype. Light blue rectangles in the second row indicate the positions of amplicons in the current on market AmplideX® Nanopore Carrier Plus Kit C. Newly added amplicons targeting *HbF* modifiers (*MYB*, *BCL11A*, and *KLF1*) and endogenous controls on additional chromosomes are not shown. Red bars indicate representative large structural variants (SVs) that the assay can differentiate based on coverage pattern changes.

## RESULTS

The redesigned prototype demonstrated high concordance in a subset cohort of 35 whole blood clinical samples and 15 reference cell lines previously characterized using the AmplideX® Nanopore Carrier Plus Kit C (RUO) assay. Of the 276 total ClinVar-annotated variants present in this cohort, 275 were detected (~99.6%), and all 25 structural variants were correctly identified (100%). One previously reported benign HBB variant in an unaffected whole blood sample could not be detected in the expanded panel design due to changes in the ROI definitions.

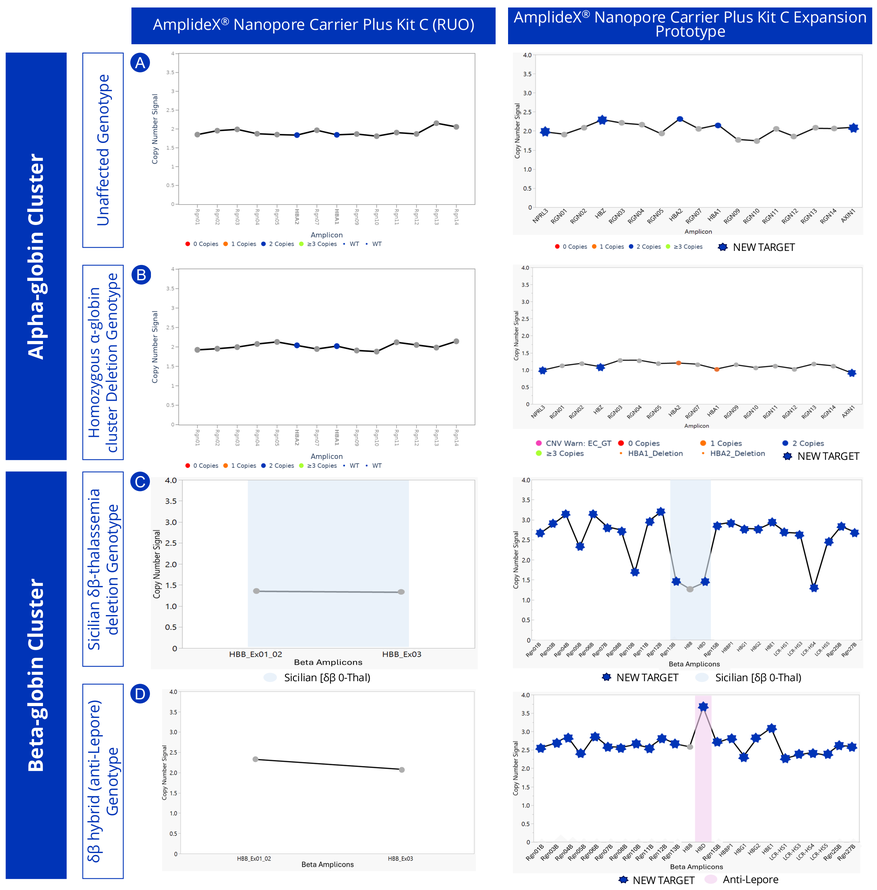


**Figure 3. Plotting Alpha and Beta-Cluster Amplicon Copy Number Signal Allows Users to Visualize Diverse Variant Genotypes.** Fifty-five total amplicons targeting the hemoglobin α-globin and β-globin gene clusters—including full gene coverage of *HBA1*, *HBA2*, and *HBB* as well as regions within *NPRL3, HBZ, HBD, HBG1, HBG2, HBE1, HBBP1, and* β-globin locus control regions— enable differentiation of common structural variants and support phasing of SNVs/indels. In addition, the incorporation of endogenous controls outside the α-globin cluster, together with α-cluster sentinel targets, improves copy-number interpretation in structurally variable regions. This design enables confident classification of clinical samples from **(A)** an unaffected whole blood sample to more challenging cases including: **(B)** a homozygous α-globin cluster deletion sample associated with coverage warnings with the AmplideX® Nanopore Carrier Plus Kit C (RUO) assay due to affected endogenous controls; **(C)** a Sicilian δβ-thalassemia deletion (13.4 kb) genotype demonstrating copy-number signal across *HBB*, *HBD*, and additional characterization across the β-globin locus control region (LCR); **(D)** an Anti-Lepore Hemoglobin Sample demonstrating an increase in *HBD* copy number signal.

| Gene | Variant Type | Unique Variants Detected | Sample Agreement for AmplideX® Nanopore Carrier Plus Kit C (RUO) | Sample Agreement for AmplideX® Nanopore Carrier Plus Kit C Expansion Prototype |
|---|---|---|---|---|
| *HBA* | Structural | SEA, FIL, THAI, 3.7del, anti-3.7, anti-4.2, and 4.2del, homozygous α-globin cluster deletion | 22/23 (95.7%) | 23/23 (100%) |
| | SNV/Indel | c.237del, c.300+64A>G | 2/2 (100%) | 2/2 (100%) |
| *HBB* | Structural | δβ hybrid (anti-Lepore) genotype, Sicilian δβ-thalassemia deletion (13.4 kb), HPFH-2, Large deletion | 3/4 (75%) | 4/4 (100%) |
| | SNV/Indel | c.20A>T, c.19G>A, c.27dup, c.316-2A>G, c.*96T>C, c.118C>T, c.126_129del, c.79G>A, c.93-21G>A, c.316-197C>T, c.217dup, | 18/18 (100%) | 18/18 (100%) |
| *KLF1* | SNV/Indel | c.803G>T[1] | 0/1 (0%) | 1/1 (100%) |

**Table 1. Pathogenic, Likely Pathogenic, and Conflicting Variant Classifications Identified in *HBA1*, *HBA2*, *HBB*, and *KLF1* Across 30 Cell-Line and 70 Whole Blood Clinical Samples.** With the expanded prototype, all *HBA1*, *HBA2*, and *HBB* variants were confirmed using orthogonal methods.

[1] The *KLF1* c.803G>T variant demonstrates conflicting clinical classifications in ClinVar and is undergoing orthogonal confirmation.

## CONCLUSIONS

- **275/276 variants (99.6%) and 25/25 structural variants (100%) previously detected using AmplideX® Nanopore Carrier Plus Kit C (RUO) were also identified with the Expansion Prototype across a subset of 15 cell-line and 35 whole-blood clinical samples, demonstrating comparable analytical performance.**
- **In addition, the Expansion Prototype identified 2 additional structural variants and multiple benign variants within newly targeted regions (*BCL11A*, *HBG1*, *KLF1*, *HBD*) that were not identified by the original assay design, suggesting data analysis would be able to identify additional pathogenic variants in these regions when present.**
- **These findings demonstrate that an expanded single-tube long-read sequencing workflow for thalassemia can improve genomic coverage across regulatory, modifier, and β-globin cluster regions while preserving the performance characteristics of the on-market AmplideX® Nanopore Carrier Plus Kit C (RUO) assay.**

**References:**

1. Seaby, E. G., & Ennis, S. (2020). Challenges in the diagnosis and discovery of rare genetic disorders using contemporary sequencing technologies. Briefings in functional genomics, 19(4), 243–258.
2. Beauchamp, K.A., Muzzey, D., Wong, K.K., Hogan, G.J., Karimi, K., Candille, S.I., Mehta, N., Mar-Heyming, R., Kaseniit, K.E., Kang, H.P., et al. (2018). Systematic Design and Comparison of Expanded Carrier Screening Panels. Genet. Med. 20, 55–63.