

SUPPLEMENTAL METHODS

PCR methods: In each assay, the PCR reaction was performed in 25 μ L volume, containing 2.5 μ L of DNA, 22.5 μ L of PCR mix including 0.25 μ L of nucleotides (dNTP Pharmacia 10 μ M final), 2.5 μ L 10x Buffer (Qiagen, Hilden Germany), 2 μ M of each primer and 1.25u Hot Star Taq Polymerase (Qiagen, Hilden Germany). A negative control (water), PCR positive control and positive control (DNA sample confirmed by Sanger sequencing) were included in each assay. Amplification of the beta globin gene was performed using forward (5'-GGT TGG GAT AAG GCT GGA TT-3') and reverse (5'-TTT GCA GCC TCA CCT TCT TT-3') primers. In some cases, Hb S mutation (Glu6Val) was evaluated by TaqMan (rs334) wherein 1 μ L of PCR product was added to 6 μ L 2x Genotyping master mix, 0.6 μ L probe mix, and water according to manufacturers' recommendation (Applied Biosystems Foster City, CA). Amplification of alpha globin genes was performed using forward primer (5'-CCC GCG CCC CAA GCA TAA AC-3') with unique reverse primers for alpha-1 (5'-CTG GCA CGT TTG CTG AGG GAA AA-3') and alpha-2 (5'-GGCACATTCCGGGATAGAGA-3').

DNA based confirmation was obtained in Hb Stanleyville II and Hb O-Arab by PCR followed by RFLP, PCR and TaqMan for G-Pest, and gap-PCR in Hb P-Nilotic and Hb Kenya. PCR primers and conditions for hemoglobin variants are included in Table S1.

Hemoglobin Stanleyville II: The diagnosis was confirmed by PCR (Table S1) and RFLP with restriction enzyme Afel. Products were digested by adding 10 μ L of PCR product to 10 μ L of digest mix [including 2 μ L 10x NEB Cut Smart Buffer, 0.4 μ L Afel (4u), New England BioLabs, Ipswich MA]. The sample was digested for 3hrs at 37°C followed by heat inactivation 75°C for 20 minutes. The samples were resolved on a 3% agarose gel

with wild type remaining uncut with a single band at 681 base pair (bp) present.

Samples that have Hb Stanleyville II will digest having a products of 483bp and 198bp, and heterozygous samples will have both uncut 681bp and cut product of 483bp and 198bp.

Hemoglobin G-Pest: The diagnosis was confirmed by PCR (Table S1) and customized TaqMan probe (rs28928875). For TaqMan, 1µl of PCR product was added to 6µl 2x Genotyping master mix, 0.6µL probe mix, and water according to manufacturers' recommendation (Applied Biosystems Foster City, CA).

Hemoglobin O-Arab: The diagnosis was confirmed by PCR (Table S1) and RFLP analysis with the restriction enzyme EcoRI (0.3u, New England Biolabs, Ipswich, MA) in a method identical to Hb Stanleyville II. The samples were resolved on a 1% agarose gel with Hb O-Arab remaining uncut and a single band at 1780bp present, while normal samples are digested into two bands of 1418bp and 362bp. Heterozygous samples will have both uncut 1780bp and digested products of 1418bp and 362bp.

Hemoglobin Kenya: The diagnosis was confirmed by Hb Kenya allele-specific gap-PCR, a method adapted from Han et al (Table S1).⁴ The PCR product was resolved on a 1% agarose gel. Both mutant (209bp) and normal allele were identified in affected samples, indicating heterozygosity for this mutation.

Hemoglobin P-Nilotic: is an anti-Lepore hemoglobin due to a β 31- δ 50 fusion. The diagnosis was confirmed by Hb P-Nilotic allele-specific gap-PCR (Table S1). The PCR product was resolved on a 1% agarose gel. Both mutant (824bp) and normal allele (923bp) were identified in affected samples, indicating heterozygosity for this mutation.

SUPPLEMENTAL TABLE LEGEND

Table S1: Summary of PCR primers and conditions for hemoglobin variants. WT; wild type.

SUPPLEMENTAL FIGURE LEGEND

Figure S1: HPLC tracings of select hemoglobin variants. There is moderate background in these samples as may be expected from aging DBS; a peak at 4.3 minutes represents post-translational modifications of Hb A, commonly seen in degraded samples. A: Hb G-Pest has similar retention time as Hb S, but in reduced quantity typical seen with alpha-chain variants. B: Hb O-Arab has the same retention time as Hb C. C: HPLC of Hb S/Kenya demonstrates that Hb Kenya has same retention time as Hb A (indistinguishable from Hb A in Hb Kenya heterozygotes). D: Hb P-Nilotic has a retention time between Hb A and Hb A2.

Variant	Mutation	Type of variant	Method of detection	Primers used	Annealing Temp	Result
Hb Stanleyville II	Asn78Lys	Alpha-chain	PCR, AfeI RFLP	Forward 5'-CAACCGTCCTGGCCCCGG-3' Reverse 5'-GGCACATTCCGGGATAGAGA-3'	62°C	WT 681 bp Stanleyville II 483bp & 198 bp
Hb G-Pest	Asp74Asn	Alpha-chain	PCR, TaqMan	Forward 5'-CCCGCGCCCCAAGCATAAAC-3' Reverse 5'-CTGGCACGTTTGCTGAGGGAAAA-3' VIC: 5'-CGCACGTGGACGACAT-3' FAM: 5'-CGCACGTGAACGACAT-3'	62°C	TaqMan
Hb $\beta^{\text{O-Arab}}$	Glu121Lys	Beta-chain	PCR, EcoRI RFLP	Forward 5'-GGTTGGGATAAGGCTGGATT-3' Reverse 5'-TTTGCAGCCTCACCTTCTTT-3'	62°C	O-Arab 1780bp WT 1418bp & 362bp
Hb Kenya	^A γ 81Leu- β 86Ala	Fusion	Gap-PCR	Forward 1 5'-ATGCCATAAAGCACCTGGATG-3' Reverse 1 5'-TCATTCGTCTGTTTCCCATT-3' Reverse 2 5'-TCCTAAGCCAGTGCCAGAAG-3'	60°C	WT 770bp Kenya 209bp
Hb P-Nilotic	β 31- δ 50	Fusion	Gap-PCR	Forward 5'-TGGTATGGGGCCAAGAGATA-3' Reverse 1 5'-TGTGGGAGAAGAGCAGGTAGGTAA-3' Reverse 2 5'-GGGGAAAGAAAACATCAAGG-3'	60°C	WT 824bp P-Nilotic 923bp

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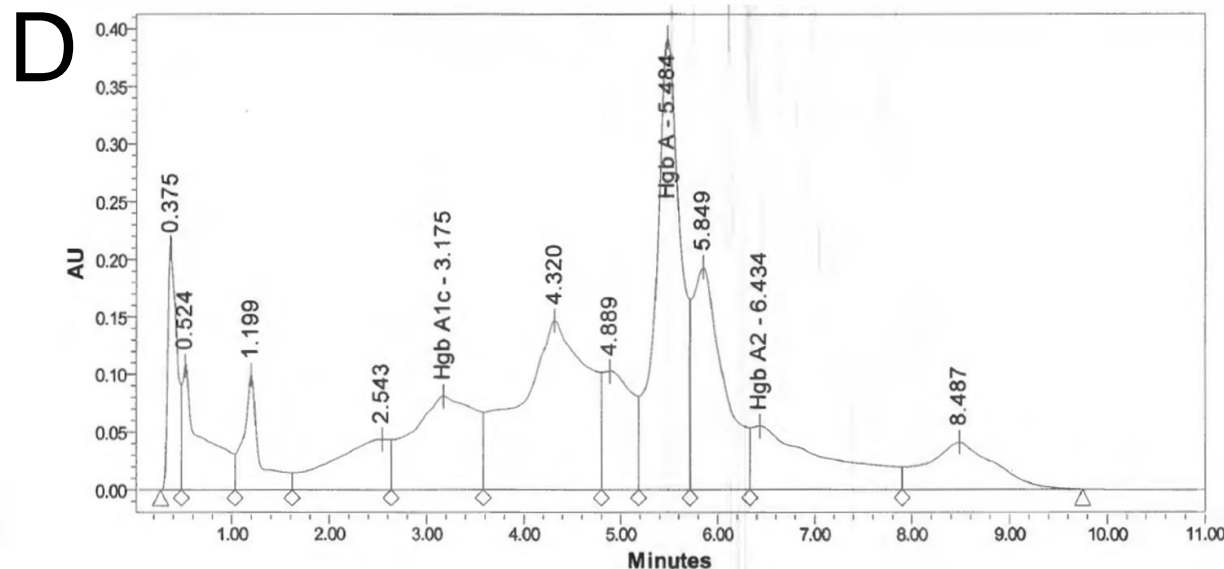
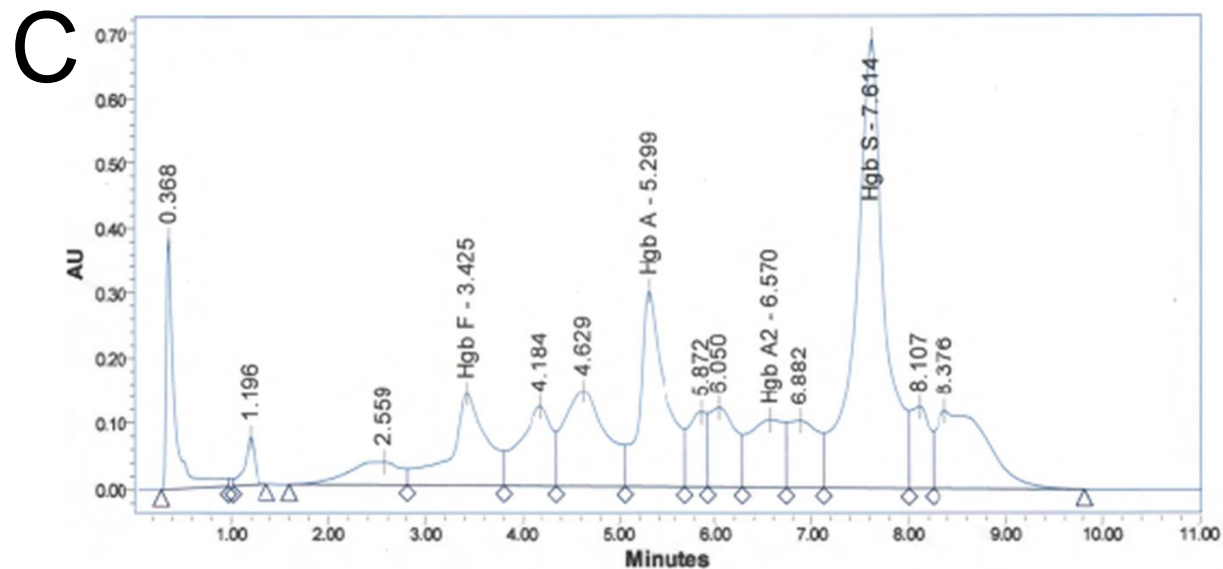
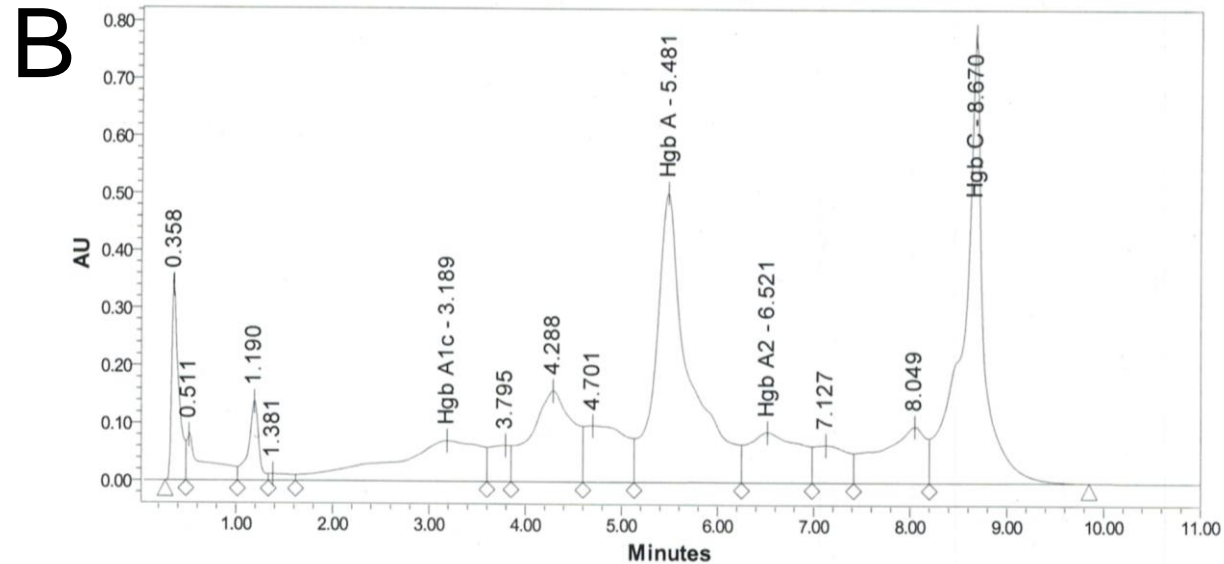
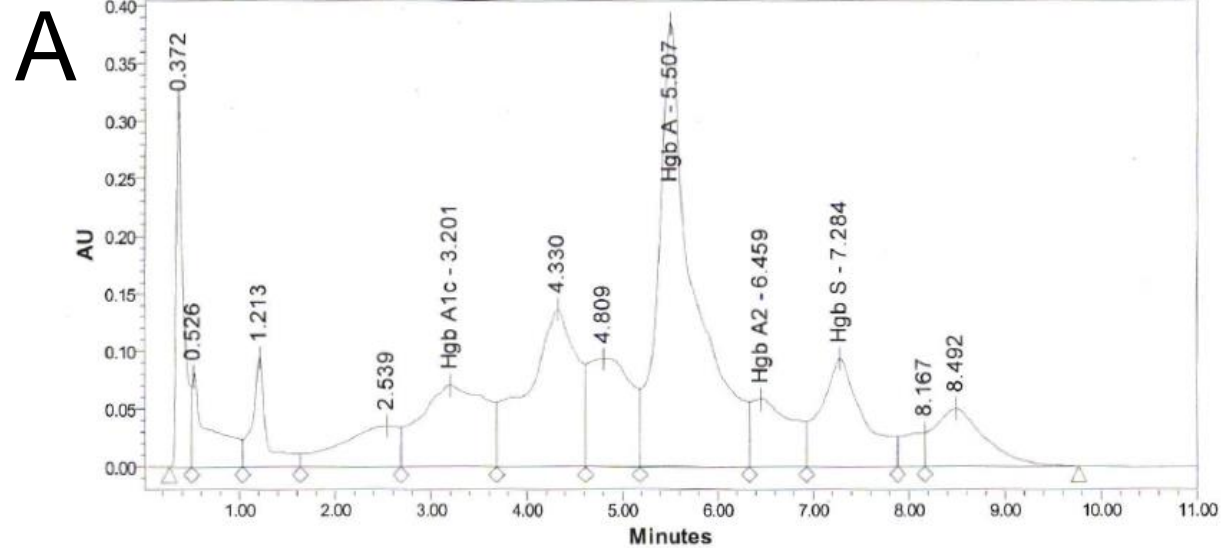


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