National Standard Operating Procedure for HPLC Result Interpretation for Hemoglobin Analysis using Bio-Rad Variant II Analyzer

First Edition/2026

Sultanate of Oman

Ministry of Health

Directorate General of Health Services and Programs

Department of Health Services Development

Diagnostic Services Development Section

Document Title	**National Standard Operating Procedure for HPLC Result Interpretation for Hemoglobin Analysis using Bio-Rad Variant II Analyzer**
Document Type	Standard Operating Procedure
Directorate/Institution	Directorate General of Health Services and Programs
Targeted Group	Primary, secondary and tertiary care laboratories
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Release Date	August 2026
Review Frequency	Every Five Years

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Date	August 2026	**Date**	August 2026

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Acknowledgments

We would like to acknowledge the team that has written the first version of this document, as well as the individuals and teams that have contributed to the revision. and lastly the taskforce members.

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Acronyms

HPLC	High Performance Liquid Chromatography
SOP	Standard operative procedure
MOH	Ministry of Health
DGHS	Director General of Health Services
RT	Retention time
IQC	Internal Quality Control
QC	Quality Control
P & P	Policy and Procedure
Hb	Hemoglobin
MCH	mean cell haemoglobin
MCV	mean cell volume
SCD	Sickle cell disease
LIS	Laboratory information system

1. **Purpose:**

   To outline the standard approach for interpreting HPLC chromatograms for the identification and quantification of hemoglobin fractions.

2. **Scope:**

   This SOP is intended for laboratory technologists and hematopathologists interpreting HPLC results.

3. **Definitions:**

   - **Bio-Rad Variant II HPLC:** An automated system used for analyzing hemoglobin variants using high-performance liquid chromatography.
   - **Chromatography:** a laboratory technique used to separate mixtures into their individual components based on differences in their physical or chemical properties.
   - **High-Performance Liquid Chromatography (HPLC):** is an analytical technique used to separate, identify, and quantify individual components in a mixture.
   - **Heterozygous**: Refers to an individual who has two different alleles for a specific gene; in the context of hemoglobin, this means one allele codes for the normal hemoglobin and the other codes for a variant hemoglobin.
   - **Homozygous**: Refers to an individual who has two identical alleles for a specific gene, in this context, both alleles code for the same hemoglobin variant.
   - **Compound Heterozygous:** Having two different mutant alleles at the same locus (e.g., results in Hb SC disease).

4. **Procedure:**

   4.1. Clinical Background: The interpretation of HPLC in hemoglobinopathies involves analyzing chromatograms to identify and quantify various hemoglobin fractions present in a patient's blood. By evaluating the retention times and peak areas, laboratory technologists and hematopathologists can detect abnormal hemoglobin variants, such as HbS, HbC, or HbE, and determine their relative concentrations. This information is crucial for diagnosing hemoglobin disorders, monitoring treatment, and guiding clinical management.

   4.2. Pre-analytical Stage: The pre-analytic stage is mentioned in Bio-Rad Variant II HPLC Analyzer Maintenance and Operation SOP

   4.3. Analytical Stage:

   - Retention Time (RT) Windows for Analytes:

Analyte	Retention Time (min)	Window (min)
F	1.10	0.98 – 1.22
P2	1.39	1.28 – 1.50
P3	1.70	1.50 – 1.90
A0	2.50	1.90 – 3.10
A2	3.60	3.30 – 3.90
D	4.10	3.90 – 4.30
S	4.50	4.30 – 4.70
C	5.10	4.90 – 5.30

- Exact RTs may slightly vary by lot, software version, and column age — always verify with control samples and retention timetable provided by Bio-Rad.
- Compare patient peaks with control/calibrator ranges; outliers require repeat testing or expert review
- Ensure peaks are within expected retention windows
- Match concentration to reference/control ranges
- Approved results should be entered into LIS - Abnormal findings must be reported promptly.
- It is preferable that a Hematopathplogist review reports before release
- When HbS is detected, a Sickle solubility test (sickledex test) must be performed for confirmation
- Iron studies (iron profile and ferritin) must be performed in presence of low MCV and MCH
- Gel electrophoresis is recommended in presence of beta chain variants (E, D, C).
- Family studies (parents testing) are recommended in all paediatric cases.

4.4. Interpretation of Results:

4.4.1 Normal Adult Hemoglobin Profile:

- HbA0: 95–98%
- HbA2: 2.4–3.4%
- HbF: <2%

4.4.2 Heterozygous β-Thalassemia

- HbA0: 90–94%
- HbA2: 3.8-8.0 % (Low MCV and MCH)

- HbF: Normal or mildly increased (1–5%)

4.4.3 Possible Heterozygous β-Thalassemia:

- HbA2: 3.8-8.0% with normal MCV and MCH
- Sample must be sent for DNA analysis to rule out other beta chain mutations.

4.4.4 Borderline HbA2:

- HbA2 value between 3- 3.7 % or > 4% regardless to red cell indices will require iron studies (Ferritin and iron profile) and genetic testing to exclude presence of milder beta thalassemia mutations.

4.4.5 Homozygous β-Thalassemia:

- HbA by markedly reduced or absent, HbA2: > 3.5 , HbF >90 %. The level of HbA varies according to the type of the mutation and age of the child. The diagnosis can be confirmed by family study or genetic testing.

4.4.6 Heterozygous Sickle Cell :

- HbA0: ~55–60%
- HbS: ~30–40%
- HbF: Normal
- HbA2: can be normal or over-estimated in presence of HbS

4.4.7 Heterozygous Sickle Cell may be coexist with Alpha Thalassemia &/or iron deficiency.

- HbA0: ~65–75%
- HbS: ~20–29%
- HbF: Normal
- HbA2: can be normal or over-estimated in presence of HbS

4.4.8 Sickle Cell Disease (Homozygous HbS)

- HbS: >50%
- HbF: variable (↑, often 5–20%)
- No HbA0
- HbS level between 41-50 % requires genetic testing to exclude presence of compound heterozygous of HbS and mild beta thalassemia mutations.

4.4.9 Compound Heterozygosity of HbS with Other Beta Variants

4.4.9.1 Compound Heterozygosity of HbS with HbC (HbS/C):

- HbS: 45–55%

- HbC: 40–50%
- HbF: 2–5% (may be increased)
- HbA0: Absent or <2%

4.4.9.2 Compound Heterozygosity of HbS with HbD (HbS/D):

- HbS: 45–55%
- HbD: 40–50%
- HbF: Variable, often 2–6%
- HbA0: Absent or <2%

4.4.9.3 Compound Heterozygosity of HbS with HbE (HbS/E):

- HbS: 30–65%
- HbE: 25–65%
- HbF: 2–15% (may be increased)
- HbA0: Absent or <2%

4.4.9.4 Compound Heterozygosity of HbS with β-thalassemia (HbS/ beta thalassemia):

- HbS: 70–90%
- HbA0: Absent
- HbA2: 5–8% (can be increased)
- HbF: 5–30% (variable, often increased)

General Interpretation: The hallmark of compound heterozygosity involving HbS and another beta globin variant is the simultaneous presence of two abnormal hemoglobin peaks with minimal or absent HbA0. The proportions may vary according to the variant involved. The hemoglobin electrophoresis and Sickle solubility test (sickledex test) are used for confirmation. DNA analysis could be considered in uncertain diagnosis after discussion with hematopathologist.

4.4.10 Heterozygous HbE:

- HbA0: ~70–75%
- HbA2 (~co-elutes with HbA2): ~25–30%
- HbF: Normal
- Please note that presence of alpha thalassemia may lower HbA2 level.
- In general, HbA2 level of >15% may indicate the presence of HbE.
- If uncertain, request DNA analysis

4.4.11 HbE Disease (HbEE):

- HbA2: ~95%
- HbA0: absent
- HbF: may vary
- DNA analysis may need to be considered to differentiate HbEE and HbE/ beta thalassemia (to be discussed with hematopathologist)

4.4.12 Heterozygous HbD:

- HbA0: ~50–60%
- HbD: ~35–40%
- HbF: Normal
- HbA2: maybe under-estimated in presence of HbD
- May co-elute near HbS window. confirm with hemoglobin electrophoresis.

4.4.13 HbD disease

- HbA0: absent or < 5%
- HbD: > 90%
- HbF: Normal or elevated
- HbA2: maybe under-estimated in presence of HbD
- May co-elute near HbS window. confirm with hemoglobin electrophoresis.

4.4.14 Heterozygous HbC :

- HbA0: ~50–60%
- HbC: ~35–40%
- HbF: Normal
- HbA2: maybe over-estimated in presence of HbC
- HbS:< 1% co-elution of HbC in S window. To be gather with HbC window.

4.4.15 HbC disease

- HbA0: absent or < 5%
- HbC: > 90%
- HbF: Normal or elevated
- HbA2: maybe normal or over-estimated in presence of HbC
- HbS: HbC can co-elution in S window. The percentage of hemoglobin eluted in S window to be gather with HbC window to get the total HbC percentage.

4.4.16 Possible α thalassaemia heterozygosity: In the absence of a variant Hb and β or δβ thalassaemia heterozygosity, α thalassaemia carrier states should be considered if the MCH is <26 pg.

4.4.17 Alpha variants: presence of multiple peaks following the normal peaks. DNA testing will be required to identify alpha variant mutations.

4.4.18 HbH disease: presence of early peak at Barts position with low HbA2 < 2%. HbH inclusion by supravital staining is used for confirmation and DNA testing.

4.4.19 Other beta variants or abnormal peak: hemoglobin electrophoresis and DNA testing will be required for identification. Expert hematopathologist advice should be considered.

4.4.20 Other conditions:

- If the Hb A2 is apparently 8% or higher on HPLC, a diagnosis of Hb Lepore should be considered, whereas an HbA2 level apparently >15% may indicate Hb E trait. Other variant hemoglobins also have a retention time similar to that of Hb A2 on HPLC.
- In the context of an MCH < 26 pg, an isolated raised HbF of ≥5% identifies possible heterozygosity for δβ thalassemia and genetic testing is required. In the presence of a normal MCH, HPFH should be considered when the Hb F is ≥10%.
- Possible heterozygosity for δβ thalassemia HbF 5-15%with low MCH, MCV.
- Possible heterozygosity for HPFH 15-30% normal MCH, MCV.

5. Responsibilities:

5.1 Laboratory Technologist:

- Operate instrument, perform maintenance, run and review QC/calibrators.
- Document and store all specimens properly.
- Adhere to procedures, report incidents, and ensure proper documentation.

5.2 Haematology Supervisor:

- Oversee test performance, maintenance compliance, IQC and EQC.
- Interpretation and reporting the HPLC results and flag the abnormal results to hematopathologist.

5.3 Quality Manager/Officer:

- Monitor compliance, maintain documentation, and follow corrective actions and EQC.

5.4 Hematopathologist/ pathologist:

- The interpretation and reporting the HPLC results.
- Decide further investigation such as DNA testing as required.

6. Documentation and Reporting

Version	Description	Review Date
01	Initial Release	August 2031

7. References:

- Bio-Rad Variant II HPLC Analyzer Operation Manual
- Bain BJ, Daniel Y, Henthorn J, de la Salle B, Hogan A, Roy NBA, Mooney C, Langabeer L, Rees DC; BSH Committee. Significant haemoglobinopathies: A guideline for screening and diagnosis: A British Society for Haematology Guideline: A British Society for Haematology Guideline. Br J Haematol. 2023 Jun;201(6):1047-1065. doi: 10.1111/bjh.18794. Epub 2023 Apr 19. PMID: 37271570.