Beta-Thal HbA_2 Quik Column® Procedure

Cat. No. 5341

Helena Laboratories

The Helena Beta-Thal HbA_2 Quik Column® Procedure is a microchromatographic methodology for the quantitation of HbA_2.

SUMMARY

The accurate quantitation of hemoglobin A_2 (HbA_2) in the clinical laboratory is essential for the differential diagnosis of several anemias and the thalassemias. Elevated HbA_2 is widely regarded as sufficient evidence for the diagnosis of β-thalassemia trait. HbA_2, however, may be normal if iron deficiency co-exists with the β–thalassemia trait. In the laboratory confirmation of the diagnosis of β-thalassemia trait, HbA_2 levels should be considered in conjunction with family history and laboratory data including serum iron and iron binding capacity, red cell morphology, hemoglobin, hematocrit, and mean corpuscular volume (MCV).[1, 2] Total HbA_2 levels expressed in mg/dL of whole blood in conjunction with the MCV have been reported as criteria for the differential diagnosis of β–thalassemia minor, iron deficiency anemia, and other hypochromic microcytic anemias.

HbA_2 has been quantitated using cellulose acetate electrophoresis followed by elution or densitometry,[3] anion exchange chromatography,[4-7] and immunochemical methods.[8, 9] Cellulose acetate electrophoresis followed by densitometry is an excellent screening method but greater accuracy is required for quantitating HbA_2. The Beta-Thal HbA_2 Quik Column procedure provides a rapid, accurate method requiring no specialized training or instrumentation.

PRINCIPLE

The Helena Beta-Thal HbA_2 Quik Column is an anion exchange chromatography method. The anion exchange resin is a preparation of cellulose covalently coupled to small positively charged molecules. The positively charged cellulose attracts negatively charged molecules. Proteins, such as the hemoglobins, contain many positive and negative charges due to the ionizing properties of the component amino acids. In the anion exchange chromatography of HbA_2, buffer and pH levels are controlled to cause different hemoglobins to possess different net negative charges. These negatively charged proteins are attracted to the positively charged cellulose and bind accordingly. Following binding, the proteins are removed selectively from the resin by altering the pH or ionic strength of the elution buffer. Due to the pH of the resin and the ionic strength of the HbA_2 Developer, HbA_2 does not bind to the positively charged cellulose and is eluted as the developer moves through the column. The other normal and most abnormal hemoglobins are retained by the resin. The HbA_2 fraction is compared to a total hemoglobin fraction by determining the absorbance of each using a spectrophotometer and then calculating the percentage of HbA_2.

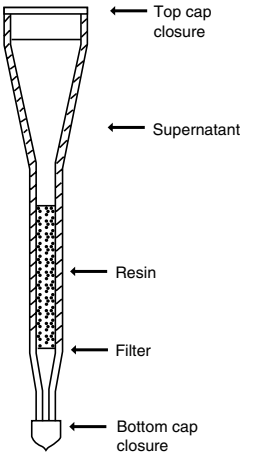
REAGENTS

1. **Beta-Thal HbA_2 QUIK COLUMNS**

   Ingredients: Each column contains ≥ 300 mg of DEAE cellulose in 0.2 M glycine buffer with 0.01% potassium cyanide and 0.1% sodium azide.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE ANY COLUMN SUPERNATANT BY MOUTH.

   This product contains Sodium Azide. To prevent the formation of toxic vapors, this reagent should not be mixed with acidic solutions. When discarding this reagent, always flush the sink with copious quantities of water. This will prevent the formation of metallic azides which, when highly concentrated in metal plumbing, may be explosive. In addition to purging pipes with water, plumbing should occasionally be decontaminated with 10% NaOH.

   Preparation for Use: Detailed instructions for preparing the columns for use are included in the STEP-BY-STEP METHOD.

   Storage and Stability: The columns should be stored at 2 to 8°C and are stable until the expiration date indicated on the box. Allow columns to warm to room temperature before use.

   Signs of Deterioration: The column should contain a slightly yellowish grey-tone resin with a clear supernatant. A vivid yellow or yellow-green color may indicate bacterial contamination.

2. **HbA_2 DEVELOPER**

   Ingredients: HbA_2 Developer contains 0.2 M glycine in deionized water. Potassium cyanide (0.01%) has been added as a preservative.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE BY MOUTH.

   Preparation for Use: The reagent is ready for use as packaged.

   Storage and Stability: The reagent should be stored at 2 to 8°C and is stable until the expiration date indicated on the bottle.

   Signs of Deterioration: The reagent should be clear, colorless solution.

3. **HEMOLYSATE REAGENT-C**

   Ingredients: Hemolysate Reagent-C is deionized water with 0.1% Triton X-100 and preservatives added.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE.

   Preparation for Use: The reagent is ready for use as packaged.

   Storage and Stability: The reagent should be stored at 2 to 8°C and is stable until the expiration date indicated on the bottle.

   Signs of Deterioration: The reagent should be a clear, colorless solution.

INSTRUMENTS

A spectrophotometer capable of reading absorbance accurately at 415 nm with a range of 0.0-2.0 absorbance (Abs) must be used. The Heme-Spec® Plus (Cat. No. 1103) is recommended.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh whole blood collected in tubes containing EDTA as an anticoagulant is the specimen of choice. Heparin or citrate tubes may also be used if desired. However, fluoride and oxalate should not be used.

Patient Preparation: No special patient preparation is necessary.

Interfering Substances: See LIMITATIONS for a complete discussion of interfering substances and other limiting factors.
Specimen Storage: The use of fresh blood samples is recommended. If necessary, specimens may be stored up to 14 days at 2 to 8°C.
Specimen Preparation: Detailed instructions for specimen preparation are included in the STEP-BY-STEP METHOD.

PROCEDURE

Materials Provided: The following materials are provided in the Beta-Thal HbA_2 Quik Column Kit (Cat. No. 5341).

50 Beta-Thal HbA_2 Quik Columns
1 x 130 mL HbA_2 Developer
1 x 20 mL Hemolysate Reagent-C

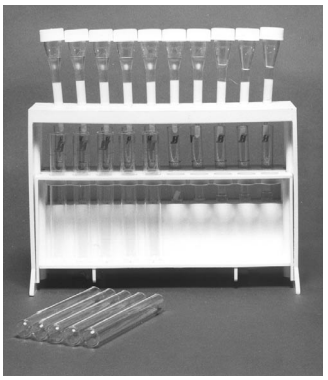
Materials Needed but not Provided:

1 Quik Column Equipment Kit (Cat. No. 5336)
1 Quik Column Rack
10 Total Fraction (TF) Collection Tubes (Large)
10 HbA_2 Collection Tubes (Small)

Pasteur pipettes with rubber bulb
Deionized Water

STEP-BY-STEP METHOD

1. For each patient quantitation to be performed obtain:
   1 Quik Column
   1 HbA_2 Collection tube (small)
   1 Total Fraction tube (large)
2. Allow the appropriate number of columns and the reagents to come to room temperature before performing the test.
3. Prepare the patient samples as follows:
   a. Whole Blood
      1) Place 50 µL of whole blood, collected in EDTA, into a small laboratory test tube.
      2) Add 250 µL of Hemolysate Reagent-C to the test tube.
      3) Vigorously shake the tube to achieve complete hemolysis of the sample. Complete lysis of the sample is essential for accurate results. If, after 5 minutes, the sample is not completely lysed, the freeze-thaw technique may be used to lyse samples.
      4) Allow the sample to stand at least 5 minutes prior to use.
   b. Packed Cells
      Alternately, saline-washed packed red blood cells may be used in the preparation of samples. To 25 µL of packed washed cells, add 300 µL of Hemolysate Reagent-C. Shake vigorously and allow to stand for 5 minutes.
4. Prepare the columns for use as follows:
   a. Upend each column twice to remove any resin from the top cap closure. Remove the top cap closure and gently resuspend the entire contents of the column using a Pasteur pipette with a small rubber bulb. Be sure all the resin is resuspended above the filter.
   b. Immediately after resuspension of each column, hold the column over a sink or absorbant paper and remove the bottom tip closure allowing the buffer to drain. If the column is allowed to stand with the bottom tip closure in place, resuspension must be repeated.
   c. As the resin repacks, you will see an interface (with a slurry above) slowly move up the tube. As soon as the slurry settles to form an interface of resin and remaining supernatant above, aspirate the undrained supernatant (making sure not to disturb the resin) and discard.
   d. Place the column in the Quik Column Rack aligned over a small collection tube.
5. Slowly and carefully apply 100 µL of the patient hemolysate to the column. During application, do not allow the sample to form bubbles or run down the side of the tube. Excessive force used during application will disturb the resin and may cause erroneous results.



6. Immediately after sample application to the column, add 100 µL of the same patient hemolysate to a Total Fraction (TF) collection tube. QS the tube to the scribed line using deionized water. Total volume = 15 mL.
7. Allow the sample to completely absorb into the resin. The hemolysate will have a glossy appearance when viewed from above until the sample is completely absorbed by the resin. Upon complete absorption, the top of the resin will have a dull, mat-like appearance.
8. Following the absorption of the sample into the resin bed, slowly apply 2.5 mL of HbA_2 Developer to the column. Excessive force when applying the developer may disturb the resin and cause erroneous results.
9. Allow all of the Developer to pass through the column into the HbA_2 (small) collection tube (approximately 30 minutes to 2 hours). The eluate contains the HbA_2. See LIMITATIONS for abnormal hemoglobins which may also elute with the HbA_2.
10. QS the small collection tube to the scribed line with deionized water. Total volume = 3 mL.
11. Invert both collection tubes several times to mix contents thoroughly.
12. Determine the HbA_2 percent in each sample using a standard spectrophotometer.
    1) Adjust the wavelength to 415 nm.
    2) Zero the instrument with deionized water.
    3) Read and record the optical density (O.D.) of both the HbA_2 and TF collection tubes.
    4) Determine the HbA_2 % as per instructions in the section entitled RESULTS: Calculation of the Unknown.

Stability of End Product: The final test solution is stable for 4 hours at room temperature.
Calibration: No calibration curve is necessary.
Quality Control: The Normal HbA_2 Quik Column Control (Cat. No. 5339) and Abnormal HbA_2 Quik Column Control (Cat. No. 5333) are available from Helena Laboratories. Controls should be run with each set of unknowns for continued quality control. The lyophilized hemolysate has been assayed for HbA_2 percentage. The controls must be reconstituted as directed in the package insert. No further dilution is necessary before application to a Quik Column.

RESULTS

Calculation of Unknown: HbA_2 may be determined using a standard spectrophotometer and performing calculations according to the formula:

$$\frac{\text{Abs of } HbA_2 \text{ Fraction}}{5\ (\text{Abs of TF Solution})} \times 100 = HbA_2\ \%$$

In the formula:
HbA_2 % = percentage of HbA_2 in the sample.
Abs of HbA_2 Fraction = absorbance of the contents of the small collection tube at 415 nm (HbA_2 fraction).

Abs of the TF Solution = absorbance of the contents of the large collection tube at 415 nm (other hemoglobin fractions).

5 = dilution factor (15 mL of TF Tube/3 mL of HbA_2 tube = 5)

100 = percentage conversion factor

Example:
A sample yielding absorbance values of 0.11 for the small tube and 1.11 for the large tube is found to have an HbA_2 percentage of 2.0% using the above formula to perform the calculation.

REFERENCE RANGE

The reference range of HbA_2 percentages using the Beta-Thal HbA_2 Quik Column methodology for normal adults has been determined. Fifty normal adults 18 years of age or older were assayed by 3 different technicians running 50 columns each. The HbA_2 expected range determined from the study is as follows:

Normal HbA_2 Range = 2.2 - 3.3%

All laboratories using the Helena Beta-Thal HbA_2 Quik Columns should establish their own normal ranges.

INTERPRETATION OF RESULTS

Reported HbA_2 percentages in normal individuals vary according to the procedure employed. Values of 1.5-3.5% have been reported in normal adults. However, no exact boundaries exist between normal and abnormal values. Results of HbA_2 assays must be interpreted in conjunction with patient history, total hemoglobin values and other clinical and laboratory findings. Any value between 3.5% and 8.0% is considered indicative of β-thalassemia trait. Values above 8% indicate the presence of additional hemoglobin variants such as HbC, E, O, D, G, S or S-G Hybrid which elute with HbA_2.

Disorder	No. Donors	Mean HbA_2%	Range HbA_2%
Individuals with normal HbA_2 level	124	2.28	1.5-3.0
β-Thalassemia Homozygous	4	3.2	2.8-3.5
β-Thalassemia Trait	45	4.97	3.5-6.3
Persistant high HbF trait	52	1.65	1.1-2.2
Hb-Lepore trait	4	2.1	1.9-2.3
Aplastic anemia	6	2.1	1.7-2.3
Hemolytic anemia	7	2.4	2.1-2.6
Leukemia	5	2.6	1.7-3.7
Megaloblastic anemia	3	3.2	3.0-3.5
Iron deficiency anemia	6	1.5	1.3-1.8
Polycythemia vera	3	2.3	2.0-2.5

Table 1: Percentages of the HbA_2 in some hematological disorders are hemoglobinopathies.[10]

PERFORMANCE CHARACTERISTICS

Precision studies were performed using two lots of Beta-Thal HbA_2 Quik Columns, two lots of HbA_2 Developer and twelve patient samples. Each sample was assayed 10 times with a mean SD of 0.124 and a mean CV of 3.25%.

The coefficient of variation obtained in lot-to-lot studies of the Beta-Thal HbA_2 Quik Columns was 2.65%.

LIMITATIONS

The results of the Beta-Thal HbA_2 Quik Column Method may be affected by the following conditions:

1. Incorrect preparation of the column
   a. Failure to completely resuspend the contents of the column may cause slow flow and erroneous results. Time must be allowed after resuspension for the formation of a distinct interface between the resin and supernatant. Any trapped bubbles may be removed with a Pasteur pipette.
   b. The bottom tip closure must be removed immediately after resuspension. Resuspension must be repeated if the column is allowed to sit with the bottom closure in place after resuspension. Failure to do so may cause slow flow and erroneous values.
   c. As soon as the resin re-packs, the remaining supernatant must be aspirated and discarded.
   d. To avoid back pressure in the column, do not remove the bottom tip closure before removing the top cap closure. Any bubbles trapped in the column resin may slow or stop the flow rate, leading to erroneous results.
2. Incorrect developer flow
   Should the developer cease to flow through the column during the procedure, the column must be discarded and the quantitation repeated with a fresh column.
3. Temperature of column and reagents
   The columns and all reagents should be equilibrated to room temperature (21 to 30°C) before running the procedure. Keep unused columns at 2 to 8°C.
4. Disturbance of resin
   Any disturbance of the resin during the procedure may cause erroneous results.
5. Resin drying out
   It is important not to allow the top of the resin to dry out before adding developer. No more than 5 minutes should elapse from the time the column stops flowing (during preparation for use) until the Developer is added.
6. Exposure of the column to extreme conditions
   The columns must not be exposed to direct sunlight or excessive heat (> 30°C) or cold (< 21°C) during the performance of the test. The column must not be frozen at any time.
7. Abnormal Hemoglobins
   a. Some of the abnormal hemoglobins (HbS,C,E,O,D, G,S-G Hybrid) are eluted with HbA_2 in this methodology. The presence of the abnormal hemoglobins should be confirmed by electrophoretic techniques. HbF does not interfere with HbA_2 quantitation.
   b. The investigator should be suspicious of the HbA_2 assay if, during the run, the Hemoglobin A band does not remain tight. This may occur if HbS is present in the sample. The resulting HbA_2 values have a tendency to run higher than actual values.
8. Incomplete Sample Absorption
   Failure to allow complete absorption of the sample into the resin before addition of the developer can be detected by a reddish tinge to the developer in the column.
9. Linearity of the Spectrophotometer
   Use of a Spectrophotometer with inadequate linearity between 1.0 O.D. and 2.0 O.D. produces high values.

TROUBLESHOOTING

Presented below is a troubleshooting guide for the Beta-Thal HbA_2 Quik Column Method. At the end of each symptom is a set of numbers in parentheses. These numbers reference more detailed explanations found in the LIMITATIONS Section of this procedure.

1) Excessive time is required for the hemolysate to absorb into the resin (> 5 minutes), or for Developer to flow through the column (approximately 30 minutes to 2 hours). (1,2)
2) Inconsistent results are obtained. (1-6)
3) Abnormally high values are obtained. (7)
4) Use of cold developer produces low values. (3)
5) Adding the developer to the columns too fast produces low values. (4,8)

BIBLIOGRAPHY

1. Ali, M.A.M., and Schwertner, E., AJCP, 63:549-553, 1975.
2. Rowley, P.T., Am J Hem 1:129-137, 1976.
3. Schmidt, R.M. and Brosious, E.M., Basic Laboratory Methods of Hemoglobinopathy Detection, DHEW Publication No. (CDC) 76-8266, 52-55, 1976.
4. Abraham, E.C. et al., Hemoglobin, 1(1):27-44, 1976-77.
5. Efremov, G.D. et al., J Lab and Clin Med, 83(4):657-664, 1974.

6. Huisman, T.H.J. et al., J Lab Clin Med, 86(4):700-702, 1975.
7. Mathews, C. and Quittner, H., Practical Chemical Hematology: Hemoglobin A_2 by Column, 16th Annual Post Grad Course in Med Tech, Royal Inland Hospital, Kamloops, B.C., 1973.
8. Chudwin, D.S. and Rucknagel, D.L., Clin Chem Acta, 50:413-418, 1974.
9. Garver, F.A. and Talmage, D.W., J Immunol Methods, 7:271-282, 1975.
10. Huisman T.H.J., Human Hemoglobins, Biochemical Methods in Red Cell Genetics, J.J. Yunis, Ed., Academic Press, Inc., New York, 424, 1969.

HbA_2 Quik Column® Equipment and Supplies

	Cat. No.
Beta-Thal HbA_2 Quik Column Kit	**5341**
Each Kit Contains:	
Beta-Thal HbA_2 Quik Columns (50)	
HbA_2 Developer (1 x 130 mL)	
Hemolysate Reagent-C (1 x 20 mL)	
Sickle-Thal Quik Column Kit	**5334**
Each Kit Contains:	
Sickle-Thal Quik Columns (25)	
HbA_2 Developer (1 x 130 mL)	
HbS Developer (1 x 300 mL)	
Hemolysate Reagent-C (1 x 20 mL)	
Quik Column Equipment Kit	**5336**
Each Kit Contains:	
Large Collection Tubes (10)	
Small Collection Tubes (10)	
Quik Column Rack (1)	

EQUIPMENT AND SUPPLIES MAY BE ORDERED SEPARATELY AS FOLLOWS

	Cat. No.
Quik Column Collection Tubes (10 small tubes, 10 large tubes)	5337
Quik Column Rack	5338
Normal HbA_2 Quik Column Control (5 x 1.0 mL)	5339
Abnormal HbA_2 Quik Column Control (5 x 1.0 mL)	5333

For Sales, Technical and Order Information and Service Assistance, call 800-231-5663 toll free.

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