Sickle-Thal Quik Column® Method

Cat. No. 5334

Helena Laboratories

The Helena Sickle-Thal Quik Column® Method is a microchromatographic methodology for the quantitation of hemoglobin A_2 (HbA_2) in the presence of hemoglobin S (HbS). The method allows for the optional determination of HbS.

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. 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 used 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 has been used to separate and quantitate HbA_2 with greater accuracy. [4-7] Immunochemical methods using antisera specific to several of the hemoglobins have been reported.[8,9] Until the introduction of the Helena HbA_2 Quik Column methodology, only electrophoretic techniques were available commercially.

The Sickle-Thal Quik Column Method is designed to quantitate HbA_2 in blood samples from individuals possessing Sickle Cell Trait. Sickle Cell Trait is the heterozygous condition in which the individual's erythrocytes contain HbA, HbS, and HbA_2. The Sickle-Thal Quik Column allows elution of HbA_2 while HbA, HbS and HbF and other abnormal hemoglobins are retained on the column. HbA_2 may also be quantitated in blood samples containing no abnormal hemoglobins. If desired, HbS maybe eluted for quantitation using an optional developer in the kit. However, HbS quantitation must be done with the understanding that other hemoglobin variants may elute as HbS and in similar concentrations.

PRINCIPLE

The Sickle-Thal Quik Column method 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 resin attracts negatively charged molecules. Hemoglobins contain many positive and negative charges due to the ionizing properties of the component amino acids. In anion exchange chromatography of hemoglobins, buffer and pH levels are controlled to cause different hemoglobins to manifest varying net negative charges. These negatively charged hemoglobins are attracted to the positively charged resin and bind accordingly. Following binding, the hemoglobins are removed selectively from the resin by altering the pH or ionic strength of the elution buffer (developer). HbA_2 and HbS are eluted with the A_2 Developer and HbS Developer, respectively. The HbA_2 fraction is eluted first and compared to the total hemoglobin by determining the absorbance of each using a spectrophotometer and the calculating the percentage of HbA_2. HbS may be quantitated in the same manner by subsequent elution from the column.

REAGENTS

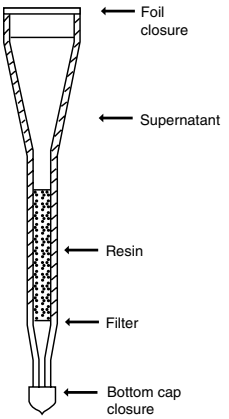
NOTE: Do not use the components of this kit with the components of any other lot numbered kit. The reagents and columns should not be interchanged between kits.

1. **Sickle-Thal Quik Column**

   Ingredients: Each column contains ≥ 600mg 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 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 stabile until the expiration date indicated on the box.

   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. **Sickle-Thal A_2 Developer**

   Ingredients: The 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 developer is ready for use as packaged.

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

   Signs of Deterioraton: The developer should be a clear, colorless solution.

3. **HbS Developer**

   Ingredients: HbS Developer contains 0.2 M glycine and 0.014 M sodium chloride 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 developer is ready for use as packaged.

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

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

4. **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 ONLY.

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 vial.

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 HemeSpec® Plus (Cat. No. 1103) is recommended.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh whole blood collected in tubes containing EDTA as anticoagulant is the specimen of choice.

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 7 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 Sickle-Thal Quik Column Kit (Cat. No. 5334).

25 Sickle-Thal Quik Columns
1 x 130 mL Sickle-Thal A_2 Developer
1 x 300 mL HbS 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 Large Collection Tubes
- 10 Small Collection Tubes

Spectrophotometer capable of reading absorbance accurately at 415 nm with a range of 0.0 - 2.0 Abs.
Pasteur pipettes with rubber bulb
Deionized Water

STEP-BY-STEP METHOD

1. For each patient or control quantitation to be performed obtain:
   1 Sickle-Thal Quik Column
   1 Small Collection Tube
   1 Large Collection Tube (2 Large Collection Tubes are needed if HbS is quantitated).
2. Equilibrate the appropriate number of columns and the reagents 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 200 µL of Hemolysate Reagent-C to the test tube.
      3) Vigorously shake the tube and allow to stand at least 5 minutes in order to achieve complete hemolysis of the erythrocytes. Complete lysis is essential for accurate results using the Sickle-Thal Quik Columns. If, after 5 minutes, the erythrocytes are not completely lysed, we recommend the use of the freeze-thaw technique.

   b. Washed Packed Cells
   Alternately, saline washed, packed red blood cells may be used in the preparation of samples. To 25 µL of washed packed cells, add 225 µL of Hemolysate Reagent-C. Shake vigorously and allow to stand for 5 minutes.
4. Prepare the Sickle-Thal Quik 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 completely resuspend the entire contents of the column using a Pasteur pipette with a small rubber bulb.
   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 elute. If the column is allowed to stand with the 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 supernatant, aspirate the remaining supernatant (making sure not to disturb the resin) and discard. It is very important to remove all the buffer from the column. Excess buffer remaining on the column will cause erroneous HbA_2 results.

5. Slowly and carefully apply 100 µL of the patient sample hemolysate to the Sickle-Thal Quik Column. During application, do not allow the sample to form bubbles or run down the side of the columns. 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 sample preparation to a large Collection Tube labeled Total Fraction (TF). 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 has a dull mat-like appearance.
8. Elution of HbA_2
   a. Following absorption of the hemolysate into the resin, place the column in the Quik Column Rack aligned over a Small Collection Tube.
   b. Slowly apply 3.0 mL of Sickle-Thal A_2 Developer to the column. Excessive force will cause disturbance of the resin, giving erroneous results. The developer remaining above the resin should be clear. If the developer contains hemoglobin, the column should be discarded and the test repeated with a fresh column.
   c. Allow all of the developer to pass through the column into the Small Collection Tube (approximately thirty minutes to one hour). The eluate contains the HbA_2. See LIMITATIONS for abnormal hemoglobins which may elute with the HbA_2.
   d. If the eluate in the Small Collection Tube does not reach the scribed line (3 mL volume), add deionized water to adjust the level to the scribed line.

9. Elution of HbS (optional)
   a. Within 5 minutes of complete elution of the A_2 Developer, place the column over a Large (15 mL) Collection Tube labeled HbS.
   b. Add a total of 10 mL of HbS Developer to the column. IMPORTANT: No more than 4.5 mL of this developer can be added to the column at one time. It is suggested that the developer be added to the column in 3 mL, 3 mL and 4 mL aliquots. It is important that the resin never remain dry for more than 5 minutes between addition of developer.
   c. When elution is complete (may take up to 2 hours), Q.S. the HbS Collection Tube to the scribed line with deionized water. Total volume = 15mL.
10. Invert all tubes slowly several times to insure thorough mixing.
11. Determine the percentage of HbA_2 and HbS using a standard spectrophotometer.
    1) Adjust the wavelength of the spectrophotometer to 415nm.
    2) Zero the instrument with deionized water.
    3) Read and record the absorbance of each eluate and each Total Fraction (TF).
    4) Determine the percent of HbA_2 (and HbS) as directed in RESULTS , Calculation of Unknown.

Stability of End Product: The final test solution is stable for 24 hours at room temperature.

Calibration: No calibration curve is necessary.

Quality Control: The HbA_2 Quik Column® Control (Cat. No. 5339) and Abnormal HbA_2 Quik Column® Control (Cat. No. 5333) are available from Helena Laboratories. A control should be run with each set of unknowns. The lyophilized hemolysate has been assayed for HbA_2 percentage. The control must be reconstituted according to the directions included with the control. No further dilution is necessary before application to the Sickle-Thal Quik Column.

RESULTS

Calculation of Unknown:

The percentage of HbA_2 present in the blood sample is determined by the following formula:

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

The percentage of HbS present in the blood sample is determined by the following formula:

$$\frac{\text{Abs of HbS Eluate}}{\text{Abs of TF Solution}} \times 100 = \text{HbS}\%$$

In the formulas:

HbA_2% = percentage of HbA_2 in the sample

HbS % = percentage of HbS in the sample

Abs of HbA_2 Eluate = Absorbance of the contents of Small Collection Tube at 415 nm (HbA_2 fraction)

Abs of TF Solution = Absorbance of the contents of the Total Fraction Large Collection Tube at 415 nm (all hemoglobins)

Abs of HbS Eluate = Absorbance of the contents of the HbS Large Collection Tube at 415 nm (HbS Fraction)

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

100 = Percentage conversion factor

EXAMPLE:

A sample yielding absorbance values of 0.187 for the HbA_2 small tube fraction, 0.300 for the HbS fraction, and 1.320 for the TF has a HbA_2 percentage of 2.8% and a Hbs percentage of 23%.

$$\frac{0.187}{5(1.320)} \times 100 = 2.8\%\ HbA_2$$

$$\frac{0.300}{1.320} \times 100 = 22.7\%\ HbS$$

LIMITATIONS

The results of the Helena Sickle-Thal 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 or similar probe.
   b. The bottom tip closure must be removed immediately after resuspension of the resin. Resuspension must be repeated if the column is allowed to stand with the bottom cap 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 procedure repeated with a fresh column. The time required for HbA_2 elution should be no longer than 1 hour and the HbS time for elution should be no longer than 2 hours.
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. Store 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 the 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 temperatures above 30°C or below 21°C during the performance of the test. The column must not be frozen at anytime.
7. Abnormal Hemoglobins
   Some of the abnormal hemoglobins (HbC,E) are eluted with HbA_2 in this methodology. Suspected presence of abnormal hemoglobins should be confirmed by electrophoretic techniques.

EXPECTED VALUES

A normal range study using the Helena Sickle-Thail Quik Column methodology was conducted. The fresh whole blood samples from 100 normal adults were quantitated according to the procedure. The following results were obtained:

Mean: 2.7%

Range: 1.7 - 3.3%

All laboratories using the Sickle-Thal Quik Column Method 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 41 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 below 1.5% are frequently associated with severe iron deficiency.[3] Values above 8% indicate the presence of additional hemoglobin variants such as HbC and E. Hemoglobins D,G and Lepore migrate in the HbS region. Patients with homozygous HbS cannot be tested with this method as it's not retained on the column.

Table 1 lists HbA_2 percentages in normal and disease states as determined by the simplified DEAE-cellulose chromatographic procedure.

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
Persistent 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 HbA_2 in some hematological disorders and hemoglobinopathies.[10]

Table 1 emphasizes the importance of HbA_2 quantitation in β–thalassemias.

Accumulating laboratory data emphasizes the increasing need for reliable HbA_2 quantitation and indicates that thalassemia genes are at least ten times more common than the sickle cell gene in the world's population. The purpose of the Quik Column is to provide the clinical laboratory with a technique to accurately measure HbA_2 values.

PERFORMANCE CHARACTERISTIC

HbA_2 Within-Run Precision:

Within-run precision of the Sickle-Thal Quik Column method for the determination of HbA_2 was determined by running blood samples from 11 donors on 10 different columns within the same run. The following mean standard deviation and mean coefficient of variation were obtained:

$$\overline{SD} = 0.062 \qquad \overline{CV} = 2.49\%$$

HbS Within-Run Precision:

Within-run precision of the Sickle-Thal Quik Column method for the determination of HbS was determined by running blood samples from 4 donors on 10 different columns within the same run. The following mean standard deviation and mean coefficient of variation were obtained:

$$\overline{SD} = 1.07 \qquad \overline{CV} = 2.96\%$$

HbA_2 Run-to-Run Precision:

Run-to-run precision studies for the determination of HbA_2 were determined by running blood samples from 6 donors one time each in 10 different runs. The following mean standard deviation and coefficient of variation were obtained:

$$\overline{SD} = 0.077 \qquad \overline{CV} = 2.89\%$$

HbS Run-to-Run Precision:

Run-to-run precision studies for the determination of HbS were determined by running blood samples from 4 donors 1 time each in 10 different runs. The following mean standard deviation and coefficient of variation were obtained:

$$\overline{SD} = 0.93 \qquad \overline{CV} = 2.67\%$$

Lot-to-Lot Reproducibility:

Lot-to-lot reproducibility was determined by running the same donor sample on 4 lots of columns. Ten tests were performed on each resin. The standard deviation and coefficient of variation were as follows:

$$SD = 0.038 \qquad CV = 1.62\%$$

BIBLIOGRAPHY

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HbA_2 Quik Column® Equipment and Supplies

Sickle-Thal Quik Column Kit **Cat. No. 5334**

Each Kit Contains:
- Sickle-Thal Quik Columns (25)
- Sickle-Thal A_2 Developer (1 x 130 mL)
- HbS Developer (1 x 300 mL)
- Hemolysate Reagent-C (1 x 20 mL)

Beta-Thal HbA_2 Quik Column Kit **Cat. No. 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)

Quik Column Equipment Kit **Cat. No. 5336**

Each Kit Contains:
- Large Collection Tubes (10)
- Small Collection Tubes (10)
- Quik Column Rack (1)

EQUIPMENT AND SUPPLIES MAYBE ORDERED SEPARATELY AS FOIIOWS

	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
HemeSpec®Plus	1103

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