I. PRINCIPLE:

The Variant™ II β-thalassemia short program is intended for the separation and area percent determinations of hemoglobins A_2 and F, and as an aid in the identification of abnormal hemoglobins in whole blood using ion-exchange high-performance liquid chromatography (HPLC).

The samples are automatically mixed and diluted on the Variant™ II's Sampling Station and injected into the analytical cartridge. The Variant™ II Chromatographic Station's dual pumps deliver a programmed buffer gradient of increasing ionic strength to the cartridge where the hemoglobins in the sample are separated based on their ionic interactions with the cartridge material. The separated components then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. An additional filter at 690nm corrects for background absorbance.

The Variant™ II Clinical Data Management (CDM) software performs reduction of raw data collected from each analysis. One level calibration is used for adjustment of the calculated HbA_2/F values. A sample report and chromatogram are generated by CDM for each sample.

Minor differences in the separation efficiency of individual analytical cartridges are corrected by the use of the Hemoglobin A_2/F Calibrator.

II. SPECIMEN COLLECTION AND HANDLING:

Specimen Type
Whole blood

Specimen Additives, Preservatives
Specimens must be collected in a vacuum tube containing EDTA.

Specimen Storage
Specimens are stable for 7 days when stored 2-8°C or 48 hours at room temperature

Specimen Rejection Criteria
Hemolyzed Samples
Samples with bilirubin greater than 20 mg/dL
Samples with triglycerides greater than 4600 mg/dL
Clotted samples and samples with volumes less than 1.5mL may be used if diluted 1:200 with wash solution prior to analysis.

III. REAGENTS

A. The Variant™ II Beta Thalassemia Short Program Reorder Pack: (Catalog #270-2103) contains supplies for 250 analysis. **Use only reagents, calibrators and cartridge lots as specified on the CD. Do not mix reagents from two different lots.** Includes:

1. Elution Buffer 1:
   Two bottles containing 1900 ml of a Sodium Phosphate buffer. Contains less than 0.05% sodium azide as a preservative. Store at room temperature.

2. Elution Buffer 2:
   One bottle containing 1800 ml of a sodium phosphate buffer. Contains less than 0.05% sodium azide as a preservative. Store at room temperature.

3. Wash/Diluent:
   One bottle containing 1800 ml of deionized water. Contains less than 0.05% sodium azide as a preservative. Store at room temperature.

4. Analytical Cartridge:
   One cation exchange analytical cartridges capable of performing up to 250 injections each. Store at room temperature.

5. HbA_2/F Calibrator with Diluent:
   One box containing six vials of lyophilized human red blood cell hemolysate containing gentamicin, tobramycin and EDTA as a preservative and one bottle diluent. Store at 2-8°C. The Diluent bottle contains 100 ml of deionized water with less than 0.05% sodium azide as a preservative. Store at 2-8°C.

6. Hemoglobin Primer Set Three boxes each containing ten vials of lyophilized human red blood cell hemolysate with gentamicin, tobramycin and EDTA as preservative. Store at 2-8ºC.

7. Sample vials, each bag contains 100 polypropylene sample vials, 1.5 ml with piercable caps.

8. CD-R, One CD with Variant II Beta –thalassemia program parameters.

B Lyphocheck A_2 Control:

IV. QUALITY ASSURANCE:

The Hemoglobin Primer Reagent and the HbA_2/F Calibrator are run at the beginning of every run.

A Lyphochek Hemoglobin Control available through Bio Rad (Catalog No.553) is run at the beginning and end of each group of patients. This is a comprehensive control product designed for monitoring the precision of automated quantitative procedures for hemoglobins A_2 and F.

Area counts for controls and calibrators must be within 1 to 3 million. The Variant™ II is programmed to halt the run if the area counts are not within 1 to 3 million. The Variant™ II is also programmed to halt if the controls are not within the defined limits or the calibration factor is not between 0.7-1.3.

The HbA_2/F Calibrator, which has assigned values (in units of area percent of total hemoglobin) for both hemoglobin A_2 and F is analyzed at the beginning of every run. Analysis of the calibrator yields separate calibration factors for both hemoglobin A_2 and F. Calibration factors are the calculated ratio of the assigned value imprinted on the labeling to the observed value (value determined per run). These calibration factors are applied to the observed area percent for hemoglobin A_2 and F in all subsequent analyses for the run. The calibration corrects for the resulting differences in the allocation of area to the hemoglobin A_2 and F peaks. The A_2 and F calibrator factors are required to be between 0.7 and 1.3.

In addition, when **a new cartridge is installed**, a normal and abnormal patient from the previous cartridge are run to compare the results and validate the new cartridge. These are filed in the notebook under the cartridge comparison. See Appendix I, How to install a new cartridge.

Patient controls SS and SC are run once a week to give a check for these abnormal hemoglobins. Delta check between the original value and the resulting value each week should be no greater than +/- 2%.

Weekly on the Variant™ II, >50% S patient sample prior to a normal patient sample to test for carryover. The normal patient sample should not have any %S carryover. If there is a %S carryover problem, patient results are with any %S are not reported from that run. The samples are repeated on the D10, and Bio-Rad service is called.

Controls and Calibrators must be run by the engineer after a PM, repair and decontamination. The engineer cannot leave until all these parameters are checked.

VI. ASSAY PROCEDURE:

A. Flush the Piston/ Seal Wash Port
   a. Fill a 10 mL syringe with DI water. Insert the syringe into the piston/seal wash port.
   b. Open the wash port $^1/_2$- turn counterclockwise
   c. Slowly depress the syringe plunger until all liquid has been dispensed. Leave the water in the lines.
   d. Close the port, turning clockwise and remove the empty syringe. Visually inspect the lines for the presence of air bubbles. Repeat a-d if present.

B. Return Variant™ II to active state
   a. Click on maintain on left hand side of home screen
   b. Click on return to active. Instrument will return to the ready state.
   c. Record injection # in log.
      i. Select setup on left of home screen
      ii. Select tests
      iii. Select cartridge

C. Prepare Reagents For Testing
   a. Whole Blood Primer
      i. Add 1.0 mL of DI water to the vial.
      ii. Allow to stand for 10 minutes at room temperature
      iii. Swirl gently to dissolve and ensure complete mixing
      iv. Add entire contents to sample vial
      v. The whole blood primer is interchangeable between lots
   b. Hemoglobin A_2/F Calibrator
      i. Reconstitute by adding 10 mL of Calibrator Diluent to each vial.
      ii. Allow to stand for 10 minutes at room temperature
      iii. Swirl gently to dissolve and ensure complete mixing
      iv. Reconstituted calibrator is stable for 10 days at 2-8 °C
      v. Add 1ml to sample vial

c. Lyphocheck A_2 Control
   i. Reconstitute Level 1&2 by adding 0.5 ml of distilled water
   ii. Allow to stand for 10 minutes at room temperature
   iii. Swirl gently to dissolve and ensure complete mixing
   iv. Reconstituted controls are stable for 21 days at 2-8 °C
   v. Add 5µL control to 1mL of wash solution into a sample vial
   vi. Vortex to ensure complete mixing

D. Prepare Samples for testing
   a. Allow the patient samples to reach room temperature and check each sample for a clot. Use a 1:200 dilution with wash solution for following types of samples:
      - **Low volume**
      - ***HCT >45***
      - ***HCT <30***
      - **Clotted samples**
      - **Microtainers**

E. Prepare run as follows:

Tube Position	**Adapter Label**	**Reagent**
1	PRIMER	Reconsistituted Whole Blood Primer (1mL)
2	BLANK	DI Water (1 mL)
3	Unlabeled	Reconstituted HbA_2/F Calibrator (1mL)
4	Calibrator Level 1	Reconstituted HbA_2/F Calibrator (1mL)
5	Control Level 1	Prediluted Control level 1
6	Control Level 2	Prediluted Control level 2
7 to N	---	Patient Samples
N+1	Control Level 1	Prediluted Control level 1
N+2	Control Level 2	Prediluted Control level 2
N+3	Stop	------

VII. RESULTING:

REFERENCE RANGES:

Normal HbA_2 2.1 to ≤ 3.9%

Normal Hb F 0 to ≤ 2.9%

Specimens which do not meet criteria for either Agar and Acetate or Resident Review may be verified by the technologist. A second technologist must double check all Variant II chromatograms prior to verification.

Agar and Acetate Reflex Criteria

1.) Any of the following:
   Hgb F ≥ 4.0%
   Hgb A2 ≥4.0%
   Any Abnormal bands
   P3 peak >6.9%
2.) And no previous complete workup by Yale-New Haven Hospital. If there is a previous complete workup, proceed to the section containing Interpretation comments for the most appropriate comment.

Resident Review Reflex Criteria

1.) Anytime the Agar and Acetate Reflex criteria are met
2.) In the absence of #1, any specimen with a MCV ≤ 77

Citrate Agar and Cellulose Acetate Reflex Procedure

1.) Add test codes for acetate and agar and interpretation (AGACE and SHINT)
2.) Receive order in SOFT-FLOW and print Flow label
3.) Place the Hgb% sticker on the HPLC report
4.) Make a smear for interpretation
5.) Place in resident box once Agar and Acetate complete

Resident Review Procedure

1.) Add the test code for interpretation (SHINT)
2.) Receive order in SOFT-FLOW and print Flow label
3.) Place the Hgb% sticker on the HPLC report
4.) Make a smear for interpretation
5.) Place in resident box

Once the Agar and acetate or Resident Review Criteria are met, the resident will write the Hgb%'s on the sticker that was placed on the HPLC printout. When the completed printout returns to the Sp Hem area, the technologist will type in the Hgb's that the resident has written

and signed off on the printout. (DO NOT enter incorrect results. Return sheet to resident if the Hgb results are incorrectly calculated)

Hemoglobin Screen Interpretation Comments (For previously worked up patients only. These are not for patients with a Resident Review ordered. These comments are to be entered under the predominant Hemoglobin fraction in the sample)

@TC01
NORMAL Hgb A and A2. There is no evidence for any abnormal hemoglobin; for example, the most common abnormal hemoglobins are not present. Results have been REVIEWED BY TECHNOLOGIST ONLY.

@TC02
Known SICKLE CELL DISEASE from previous Hgb analysis. Results have been REVIEWED BY TECHNOLOGIST ONLY.
Example Hgb SS, SC or S/beta thal

@TC03
Known HETEROZYGOUS for hemoglobinopathy from previous Hgb analysis. Results have been REVIEWED BY TECHNOLOGIST ONLY.
Example AS, AC, AE or AD

@TC04
Known Beta-Thalassemia Trait from previous Hgb analysis; the current elevated A2 and low MCV are consistent with this diagnosis. Results have been REVIEWED BY TECHNOLOGIST ONLY.

@TC05
Known HOMOZYGOUS for non-sickle hemoglobinopathy from previous Hgb analysis. Results have been REVIEWED BY TECHNOLOGIST ONLY.
Example: CC, DD, EE

IX. LIMITATIONS OF PROCEDURE:

-Elevated levels of A_2 may be masked by concurrent iron deficiency anemia.
-Hemoglobin S and other late eluting hemoglobin variants have minor component peaks that may coelute with HbA_2. This may result in a falsely elevated area percent value for HbA_2.
-Hemoglobins D and E have been observed to coelute with HbA_2.
-Hemoglobins Bart's and H elute prior to the start of integration
-Diabetic specimens typically exhibit an elevated P2 peak. An additional peak, identified as "Unknown", may also be present between the normal positions of F and P2. If

this additional peak elutes within the F window when there is no HbF present, it may be misidentified as HbF.

X. CLINICAL SIGNIFICANCE:

A frequently occurring thalassemia, β – thalassemia is commonly found in the heterozygous state as β-thalassemia minor or β-thalassemia trait. Carriers of the β–thalassemia gene may be afflicted with a mild anemia, or may be asymptomatic. Clinical identification of these carriers is important: any offspring between individuals with β-thalassemia trait are at risk of being homozygous for the β-thalassemia gene. The homozygous state, Beta-thalassemia major, is a lethal disease for which there is no adequate treatment. Children afflicted with Beta-thalassemia major suffer from severe anemia, jaundice, splenomegaly, bone malformations, growth retardation and usually death before maturity.

Adult blood contains primarily hemoglobin A, a small percentage of hemoglobin A_2 and trace amounts of fetal hemoglobin. Carriers of β-thalassemia have levels of hemoglobin A_2 and F, which can be greater than 3.5% and 2% of the total hemoglobin, respectively. Determination of concurrently elevated levels of hemoglobin A_2 and F has become the most practical means to diagnose carriers of the β-thalassemia gene.

APPENDIX 1

CHANGING A NEW CARTRIDGE

V2-Bthal test should be indicted in the Setup/Test box
Install a new cartridge (the arrow goes up)
Replace all reagents 1,2,&3
Do a short system flush so the reagents will cycle through the cartridge which will take about 20 minutes. (Set up- test-reagents-short flush)

Place the new CD into the CD drive. Making sure that the regent 1&2 cartridge and calibrator set lot numbers match the numbers on the CD. Select Setup/Test and Up Date kit in the upper right corner. Then select **E** disk. The CD will be automatically loaded.

Select Setup/Sample types/Patient
Check that the normal ranges are correct. They do change with a new CD.

Select Calibrator
1.Delta Factor is enabled
2.Values are correct for the new calibrator
3.Stop worklist is selected for Action if Outside of Limits on the Calibrator

Select Low control
1.Insert values (**mean and ranges that have been determined by our laboratory**) and expiration date for the control (A2&F)
2.Select stop worklist

Select High control
1.Insert values (**mean and ranges that have been determined by our laboratory**) and expiration date for the control (A2&F)
2.Select stop worklist

Select Primer
1.Insert primer lot number and expiration date

Set up sample rack according to New Cartridge directions
Place sample rack onto the Sampling Station and access Run/Worklist/Start

Note retention time for Hb A2 from Vial 6

Refer to the lot specific Release Notes, provided in each reorder pack, and compare retention time to the optimum retention time.

If retention time varies by more than 0.05 minutes from the optimum, and adjustment of the thermomodule temperature is recommended. Adjust the temperature in the Setup/Test/Cartridge screen.

If the retention time varies by more than 0.20 minutes, monitor the pump performance by measuring the flow rates and observing the pressure readings. Notify a supervisor or call Technical Service for additional instructions.

In addition, when **a new cartridge is installed**, a normal and abnormal patient from the previous cartridge are run to compare the results and validate the new cartridge. These are filed in the notebook under the cartridge comparison. See Appendix I, How to install a new cartridge.

NEW CARTRIDGE

SAMPLE NUMBER	SAMPLE TYPE
1	Whole Blood Primer
2	Whole Blood Primer
3	Deionized Water
4	Deionized Water
5	A2/F Calibrator
6	A2/F Calibrator
7	Stop Tube

APPENDIX 2

Clean and Decontamination the Sampling Fluid Path on the Variant II

Clean and decontaminate the line once a month.

1. Disconnect cartridge to detector tubing from the cartridge holder.
2. Remove the analytical cartridge (place covers on both ends of the cartridge.)
3. Clean the cartridge holder with a cotton swab and DI water. Replace it with the plastic PEEK dummy cartridge. (Located in box on shelf in a manila envelope.)
4. Install decon tubing to the outlet of the cartridge holder. Place outlet side of decon tubing into a beaker to collect waste from the decontamination procedure.
5. Select the V2 DECON test from the setup/test screen.
6. Place 5 sample micro vials of full strength bleach solution into the adapters in the first five positions of a sample rack.
7. In the last five positions of the rack, place 5 sample micro vials filled with distilled water.
8. Put the STOP tube into the second sample rack and place both sample racks on the sampling station.
9. Start the work list.
10. When the status returns to Ready, remove the plastic PEEK dummy cartridge, clean the cartridge holder again with a cotton swab and DI water, then reinstall original analytical cartridge.
11. Remove decon tubing from the cartridge holder. Discard beaker waste.
12. Connect cartridge to detector tubing, securing both tubing end connections.
13. Check off decontamination procedure with the date and initials in the daily maintenance log.

XI. REFERENCES:

1. Variant™ II β-thalassemia Short Program Instruction Manual: Bio–Rad Laboratories, November 2011.

XII. HISTORY:

H-1 This procedure was written by P. Morris on 12-99.
H-2 Revised by P Morris 11/19/10.
H-3 This procedure was revised by Susan Richardson on 5/23/12.
H-4 This procedure was revised by Andrew Link on 04/09/2013