

Appendix 5I

**Automated High Pressure Liquid Chromatography
Screening Assay for Detection of Hemoglobins
Tracking No. CN 001
Version No. 5.2**

Table of Contents

I.	Title-----	1
II.	Principle-----	1
III.	Specimen Collection and Type-----	2
IV.	Equipment and Supplies-----	2
	A. Equipment-----	2
	B. Supplies-----	3
V.	Reagents-----	3
	A. Manufactured and supplied by Bio-Rad Laboratories-----	3
	B. Manufactured and supplied by GDL-----	4
	C. Supplied by the screening laboratory-----	4
VI.	Calibration and Quality Control-----	4
	A. Calibration-----	4
	B. Quality Control-----	5
VII.	Procedures-----	5
	A. Preparation for analysis-----	5
	B. Sample Handling-----	13
	C. Prepare the Calib Tray_____	13
	D. Preparation of Sample Trays_____	15
	E. Import Worklist_____	15
	F. Create a Manual Worklist, Backup Procedure_____	19
	G. Load Plates_____	20
	H. Set up Printing_____	20
	I. Analysis_____	21
	J. Hb Pattern of F Only, No Peak, Low Area, High Area, or Fast and F1 > 50%_____	25
	K. Shutdown Procedures_____	26
	L. Repeat Testing_____	27
	M. Interrupted Run_____	27
	N. Backup Daily_____	28
	O. View Chromatograms from previous runs_____	29
	P. Maintenance_____	29
	Q. Hard Drive Restoration_____	35
	R. Troubleshooting_____	35

VIII.	Calculations-----	35
IX.	Reporting Results-----	36
	A. Quality Control Program-----	36
	B. Reporting Results-----	36
X.	Procedure Notes-----	37
XI.	Limitations of Procedure-----	37
XII.	References-----	37
Attachments	Attachment 1---Quality Control Rules for Analysis-----	38
	Attachment 2---Quality Control Action Chart-----	43
	Attachment 3---Action Limits-----	44
	Attachment 4---Weekly Preventive Maintenance Records-----	46
	Attachment 5---Monthly Preventive Maintenance Records-----	47
	Attachment 6---Instruction for Bio-Rad Service-----	48
	Attachment 7---Hemoglobinopathies Checklist-----	49
	Attachment 8---Troubleshooting Common Assay Problems -----	51

I. Title

Automated High Pressure Liquid Chromatography Screening Assay for Detection of Hemoglobins

II. Principle

Hemoglobinopathies screening is performed on all newborns as part of the California Newborn Screening Program. Presence or absence of hemoglobins F, A, E, D, S, and C is determined using a fast and automated high pressure liquid chromatography system manufactured by Bio-Rad Laboratories.

The sample for this assay is the eluate extracted in 425 μ L of distilled water from one 6 mm disk, punched from blood spots dried on filter paper. This is the eluate for the Transferase, Biotinidase, and Hemoglobin assays. The eluate is distributed into microtiter plates for testing.

The hemoglobins are eluted, diluted, and injected onto a cation exchange resin column for separation under controlled temperature and gradient. Two pumps, controlled by a process controller, generate the defined gradient. After separation the hemoglobins pass from the column to the detector. The absorbance reading from the spectrophotometer is converted to an electronic signal in millivolts and plotted versus time in minutes. The area under the curve is integrated, and the peaks are identified by their retention times. The area units under each peak are then expressed as area %. The result is a hemoglobin pattern where the hemoglobins detected are listed in order of decreasing concentration.

The method described herein is the screening method used by laboratories contracted by the State of California to determine hemoglobin patterns using blood spots collected from newborns at the time of discharge from hospitals.

III. Specimen Collection and Type

The blood spots are collected from a heel stick puncture and air-dried on the filter paper. The collection procedure is performed on the newborn at the time of discharge from the hospital but no later than 6 days of life. The dried specimen is transported to one of the State's contracted laboratories immediately after collection.

IV. Equipment and Supplies

A. Equipment

1. High pressure liquid chromatography system, Newborn Hemoglobin System (NHS 3.0). The system has
 - a. Process controller
 - 1) HP RP 5000 Window XP SP 2
 - 2) ≥ 192 MB RAM, ≥ 40 GB hard disk, 1.4 MB floppy disk drive
 - 3) Internal Read-Writable CD-ROM
 - b. High resolution color monitor
 - c. Keyboard hutch
 - d. Smart UPS
 - e. Newborn Chromatographic Station (NCS), 1 or 3 2-pump gradient system, 2 pumps, flow rate: 0.01 mL/min increments from 0.102 to 3 mL/min
 - f. HPLC column, cation exchange, 4 cm mini column, Bio-Rad, P/N 250 0014
 - g. HPLC dummy column, 3 cm PEEK cartridge, Bio-Rad, P/N 270 0220

- h. Column heater, temperature controlled, performance range: 35° C ± 0.5° C
- i. Detector, dual wavelength, 415 nm and 690 nm
- j. Newborn Autosampler (NAS), with cooling unit and injection loop. Cooling unit temperature range: 2-35° C, maintains sample tray below 13°C. Injection loop size: 23 µL.
- k. Printer

B. Supplies

- 1. Supplied by the screening laboratory
 - a. Printer paper
 - b. Printer toner
 - c. 25 and 50 mL plastic syringes
 - d. Volumetric pipettes, Class A, TD, 1 and 10 ml
 - e. Beakers, 250 mL
 - f. Kimwipe lab wipers
 - g. CD-R, 8X or greater
 - h. Eppendorf Pipettors and tips 150 uL
 - i. Falcon Trays and covers – 96 well plates
 - j. Floppy disks
 - k. Cotton swabs and wire for cleaning probe

V. Reagents**A. Manufactured and supplied by Bio-Rad Laboratories**

- 1. Buffer 1, phosphate buffer, 2000 mL/bottle, and contains sodium azide
- 2. Buffer 2, phosphate buffer, 1800 mL/bottle, and contains sodium azide
- 3. Wash solution, 900 mL/bottle, and contains sodium azide
- 4. Column primer, lyophilized whole blood, 1 mL/vial
- 5. Calibrators, 3 each, containing Hb F at concentrations of x, 2x, 3x

- a. Neonatal Linearity Standard Level 1, FA, Hb A and low concentrations of F, lyophilized from red cell hemolysates, 10 mL/vial
 - b. Neonatal Linearity Standard Level 2, FA, Hb A and medium concentrations of F, lyophilized from red cell hemolysates, 10 mL/vial
 - c. Neonatal Linearity Standard Level 3, FA, Hb A and high concentrations of F, lyophilized from red cell hemolysates, 10 mL/vial
6. System Controls, 2 each, containing Hb FAES and FADC
- a. Neonatal System Control I, FAES, lyophilized from red cell hemolysates, 10 mL/vial
 - b. Neonatal System Control II, FADC, lyophilized from red cell hemolysates, 10 mL/vial
- B. Manufactured and supplied by GDL
1. Blood spot tray quality control
 2. Blood spot proficiency control
- C. Supplied by the screening laboratory
1. Distilled water, or deionized water in conformance to the specifications for NCCLS Type I water.
 2. Bleach

VI. Calibration and Quality Control

A. Calibration

Each system used is calibrated once at the beginning of the day. The calibration tray consists of whole blood primer, three linearity standards of low, medium and high concentration of Hb F and two system controls. The low, medium and high

standards contain approximately 650,000, 1,300,000, and 1,950,000 area units of Hb F, respectively. At least two standards out of three must be within limits for the run to be valid.

B. Quality Control

Tray Controls (CT and CP), Positional, and System Controls are used as quality control samples for this assay. The system controls are used to verify that the system can recognize hemoglobin E, S, D and C accurately and reproducibly. Each calibration tray has two system control cups, containing Hb FAES and FADC.

Each tray has three tray QCs: in position 16, 49, and 96 for tray # 1, and positions 1, 49, and 96 for tray #2 and all subsequent full trays. Positional controls are water blanks located in positions 25, 26, 27, and 28 for tray #1, # 2, # 3, and # 4, respectively.

VII. Procedures

A. Preparation for analysis

1. Turn on power to PC and monitor first.
2. Turn on power to the NAS and NCS systems.
3. Adjust the brightness of the monitor.
4. Use **Ctrl Alt Delete** to logon. The screen shows user name and password. No entry is needed; it is needed only for Administrator. Just click **OK**.
5. Double Click **GDM 3.0**, Genetic Data Management. On the screen in the upper left corner is the message, "GDM is loading. Please wait." You must wait patiently until all software has been loaded. Otherwise, you may not have the entire package. **NOTE:** If power is not turned on to the NAS and NCS, error message will appear, "GDM instrument

communication problems”. To warm up each system, click on icon for each system with Instrument #.

- a. Click **Return to Active**.

The pump starts and the system starts warming up. Monitor the temperature on the bottom of the screen. At this time, the Instrument is in the **Ready state** after the power is turned on. The **Ready state** will appear on the Instrument icon even if the temperatures are outside acceptable limits. **“Cartridge temperature is outside the allowed range. OK”** appears if attempt is made to start run before temperatures are within limits.

- b. To have the NHS programmed for automatic warm-up daily, keep power on.

- 1) Click **Setup/Configuration**.

- 2) Select **Automatic Warming Up**.

- 3) Enter time (military clock) and days for automatic warm-up. Each morning, your computer screen is in GDM and the temperature of each system is 35 +/- 0.5 degree.

6. Record temperature on the Checklist.

7. Verify that each system is setup up to do newborn Hb. Click **Setup/Test**. Select **NeoHb** from the drop down menu for **Select New Test**. This is already selected for you. Use this instruction only when

needed.

8. Enter your laboratory ID. Click **Setup/Configuration** enter **Site Number** using 11, 12, 21, etc.
9. Check the wash solution level and for air bubbles in the lines for each system. Approximately 0.5 mL of wash solution is required per analysis.

You must remember to determine sufficient volumes for the number of samples tested on each NAS/NCS unit and not for the run.
10. Check the buffer solution levels and for air bubbles in the lines for each system. Ensure an adequate supply for the anticipated run size per system. Approximately 5-6 mL of Buffer 1 and 3 mL of Buffer 2 are required per analysis. The exact volume varies with each lot of reagent. If volume is 20% of total by weight, system will provide a warning.

See chart below for volumes needed using 5 mL for Buffer 1.

Number of Samples	ML Needed		
	Buffer 1	Buffer 2	Wash Sol
50	250	150	25
100	500	300	50
150	750	450	75
200	1000	600	100
250	1250	750	125
300	1500	900	150
350	1750	1050	175
400	2000	1200	200

- a. If air bubbles are present
 - 1) Open pump inlet port of appropriate system.
 - 2) Use syringe to draw buffer till no air bubbles are present.

- b. To change the buffers follow the procedure below:
- 1) Select the instrument that needs to have the buffer changed. Click Maintain/Instruments/Stop Flow.
 - 2) Pull the tubing with the fritted filter out of the reagent bottle and immediately place it into the new bottle. DO NOT lay the filter on a surface. Do not completely tighten cap on bottle.
 - 3) Draw buffer through the pumps. Open the pump inlet port by turning counterclockwise. Attach a 25 cc plastic syringe to the pump inlet port. Pull back on the syringe plunger slowly to remove 25 mL of liquid from the port. Dispose of liquid and repeat to remove a total of about 50 mL. Remove the syringe. Close the port by turning clockwise.
 - 4) Repeat for the other pump if needed.
 - 5) Discard any buffer bottle if the volume is so low that it is not sufficient for another run. Do not combine buffers. However, wash solutions may be combined.
 - 6) Run the pumps when you have allowed a **reagent bottle to run dry**, or there is other evidence that the procedure is needed. Air in the pumps in the NCS is suggested when there is significant pressure fluctuation or low pressure levels
 - a) Click Maintain/Instruments and select Stop Flow.
 - b) Remove 50 mL from the pump inlet port as above.

You should observe bubbles flowing into the

syringe. If bubbles are observed repeat the procedure an additional time to assure all bubbles have been removed.

- c) Repeat for the 2nd pump inlet port if needed.
 - d) Click **Maintain/Instruments**.
 - e) Click **Open Purge Valve**.
 - f) Set %B to 0% and **Flow Timer** to 10 minutes.
 - g) Select **Start Flow** to begin manual pump mode of Buffer1.

Run for 10 minutes. Note significant pressure fluctuation or low pressure levels at the bottom of the monitor screen.
 - h) Set %B to 100% and select **Start Flow** to begin manual pump mode of Buffer 2. Note significant pressure fluctuation or low pressure levels.
 - i) Click **Close Purge Valve**.
- 7) Click **Setup/Test** to change to buffer Lot # when a new lot is used.
- a) Select the radio button **Reagents**. On the screen are Reagent names (Buffer A, Buffer B, Wash Solution), **Lot #**, **Expiration** Date, Start Date, End Date and In Use.
 - b) Change **Yes** for **In Use** to **No** for the current lot to

have a new entry line. **End Date** is automatically entered.

- c) Enter **Lot #** and **Expiration Date** for each reagent..
- d) Change **No** to **Yes** for **In Use** and the **Start Date** is automatically entered.

- 11. Check cartridge injection number.
 - a. Select an instrument. **Click Setup/Test**.
 - b. Select the radio button for **Cartridges**.

NOTE: Access to radio buttons **Peak Table** and **Integration Table** is read only. To make changes, which you will never need to do, you need a password which you will never get.

Information on the screen includes **Lot #**, **Serial #**, **Inj(ection) Count**, **Inj(ection) Limit**, **Start Date**, **End Date**, and **In Use**.

- c. Determine, for the current column (by the **Yes** for **In Use**), if the injection count will exceed 500 during the run using the number in the **Inj Count** box. Each column is good for 500 injections. If needed, replace the column as described below. Label and save the partially used column for another day with fewer samples. You can also click the **Print** icon to print the screen to have the **Inj Count** of the partially used column.
- d. Create a new entry line by changing the **Yes** for **In Use** to **No** for the current column in used. **End Date** is automatically entered. Enter **Lot #** and **Serial #** with each new column installed. Click **Yes** for **In Use** for the new column. The **Inj Count** box goes to 0 and **Inj Limit** goes to 500. If the column is not new, enter the # of

injections for the column in the **Inj Count** box. This is for your information. The systems do not track correct use of lots.

e. Repeat for each system.

f. To change column,

- 1) Click **Maintain/Instruments** and select **Stop Flow**.
- 2) Open the column oven door.
- 3) Unscrew and separate the two pieces of the metal column holder.
- 4) Pull out the old and insert new column cartridge with the arrow pointing up, towards the low pressure end in the direction of flow.
- 5) Screw the metal holder till finger-tight. You will not damage the column seals when tightening by hand. DO NOT use tools to tighten.
- 6) Place the metal column holder onto the column oven, taking care to rest the solvent lines in the grooves.

Close the column oven door and tighten the screw until finger tight.

12. Check STD, SQC, and TQC lot numbers for system by clicking **Setup/Sample Types**. On screen are lot numbers and expiration date for Primer, Linearity Standards 1-3, FAES Controls, FADC Controls, and Tray Controls. Enter **Lot Number** and **Expiration Date** for new lot of Linearity STDs, SQC, or TQC. This is for your information. The systems do not track lot numbers.

13. Check the printer paper supply and the toner cartridge quality.

14. Check the level of the waste container. Empty if necessary and dispose properly. Add bleach to the empty container.
15. Check fittings for leaks.
16. Check each Autosampler probe to be sure it is not bent or damaged.

Prior to the start of the run, the software automatically sends the probe to home and base to verify that the arm and probe are in the correct start position. To do this when needed, select the system, click **Maintain/Instrument/Go to Base/Go to Home**.

(The **Wash** position is behind the **Home** position.)
17. Flush the piston/seal valve.
 - a. Attach a 10 mL syringe filled with distilled water to the piston/seal valve.
 - b. Depress the syringe plunger slowly until all liquid has been dispensed. Leave the water in the lines.
 - c. Remove the syringe
18. Flush the injector. Click **Maintain /Instruments**. Change **Flush Volume** to 4.0 mL. Click **Flush**. This takes about 2 – 3 minutes.
19. Start the pumps. Select an instrument, click **Maintain/Instruments**.

Flow rate is set at 2mL/min. Change **Flow Timer** to 5.00 to pump for 5 minutes. Click **Start Flow**. A timer will appear counting down from 5 minutes that disappear when completed. Status of the system is **Manual**.

Monitor the pressure. Pressure must be constant between 10 –40 kg/cm square, ideally around 20 kg/cm square. If there is wide fluctuation, it is an indication of air or leak in the system. Correct the problem using Steps

10.b.6.) above.

20. Click **Maintain/Monitor** to monitor the baseline when the pumps are on. Click **Set Scale**. Enter 0.6 volts for **Maximum**, -0.1 volts for **Minimum**, 3.00 minutes for **Duration**. Click **OK**. Click **Start**.
21. Prime the column. You must prime the column before starting the run. This takes about 10 minutes. The primer is in cup 1 of the Calib Tray.
 - a. Ensure that the system is in the **Ready** mode. If not, double click the **Instrument** icon and select **Return to Ready State/OK** or click **Maintain/Instruments/Return to Ready State**.
 - b. Click **Run/Worklist** and select **Inject Primer**. Message that the primer is in Cal tray, well 1 appears. Click **OK**. In the upper right corner is the message, "Inject Primer is performing now."
 - c. Click **OK** when the message appears on the screen, "Instrument # The Inject Primer action has finished". Second message, "The instrument is now ready", appears and disappears. Status of the system is **Ready**.
 - d. Repeat for each system used for the run. This can be done simultaneously for the 3 columns.

B. Sample Handling

The sample for this assay is the shared eluate extracted in 425 μL of distilled water from one 6 mm disk, punched from dried blood spots used for the Hemoglobin, Biotinadase, and Transferase assays. The specimens are barcoded and processed through the Specimen Gate workstation, an automated system for positive specimen identification, punching, eluting, and distributing. The eluate is distributed into microtiter plates for TRA, BIO and Hb testing. For hemoglobin testing, 38 μL of eluate is diluted with 176 μL of water. The microtiter plates, a printed worklist, and a diskette are delivered to the Hemoglobin work station.

NOTE: For details, refer to the NBS Accession and Reporting for NAPS Labs and Specimen Handling, the Processing of Blood Spots Using NeoGo protocols.

C. Prepare the Calib Tray

1 .Reconstitute the column primer, linearity standards, and system controls .

a. Reconstitute the primer with 1 mL of distilled water using 1 mL volumetric pipet. Let it stand for 15 minutes. Invert and mix. Stable for 2 weeks after reconstitution when refrigerated.

b. Reconstitute linearity standards 1, 2, and 3 and System Controls I and II with 10 mL of distilled water using a 10 mL volumetric pipet. Let it stand for 15 minutes. Invert and mix. Stable for 4 weeks after reconstitution when refrigerated.

2. Set up Calib Tray

a. Label a microplate for each HPLC system used for the run.

b. Use 150 μ L pipettor and deliver into the wells of the microplate.

Follow the format shown below:

Well #	Sample
1	Primer
2	Distilled water
3	Distilled water
4	Linearity Standard 1
5	Linearity Standard 2
6	Linearity Standard 3
7	System Control I, FAES
8	System Control II, FADC
85	Distilled water
86	System Control I, FAES
87	System Control II, FADC

CAUTION: The end of the pipette can become contaminated. The primer or other liquid can splatter onto the end of the pipette. If blood or other liquid splatters onto the end of the pipette, wipe it clean before proceeding to the next sample.

- c. Cover the trays; begin the analysis within 30 minutes. Otherwise, refrigerate the covered trays (2 to 8 °C) until analysis.
- d. Recap and refrigerate the reconstituted reagents.

D. Preparation of Sample Trays

Apricot System is used for dilution and distribution of the eluate for hemoglobin testing. Refer to protocol for Specimen Handling, the Processing of Blood Spots Using Specimen Gate for Newborn Screening, tracking number CN 003, version 7.1. For hemoglobin testing, sample trays are available after dilution and distribution by the Apricot system with the printed worklist and diskette. The microtiter plate has 96 wells. Label each microplate for hemoglobin testing with date, tray # and run #.

Sample tray 1 contains

Blood spot tray QC in cup#16
Positional QC in cup # 25
Water in cup #95
76 newborn samples
Blood spot tray QC in cup # 49 and 96.

Sample tray 2 contains

Blood spot tray QC in cup # 1
Positional QC in cup # 26
Water in cup # 95
91 newborn samples
Blood spot tray QC in cup # 49 and 96

For trays 3 and tray 4, positional QCs are in cup # 27 and cup #28 respectively.

E. Import Worklist

Do not import the worklist until **Inject Primer** is completed. The worklist wizard directs the user through importing a worklist from a diskette.

NOTE: To accommodate a software quirk, you must set up the worklist with a water cup before the last tray control of the run. If by chance the last patient sample is in the well just before the Positional Control, a water cup must be placed after the PQC and before the tray control. (The function of the PQC is different from a water cup, therefore, are not treated by the software in the same way.) The software will not transmit the last patient result if a water cup is not added. The software treats the last patient sample as the PQC and the PQC as the water cup.

1. Click **Maintain/Instruments/Return to Ready State** to bring the status of the system to the ready mode.
2. Select an instrument. Click **RUN/Worklist**. Click **Clear Worklist** if there is a worklist for the system. All lines to the worklist should be grey indicating that all samples had been injected. Click **Clear** when the prompt, "Clear worklist?" appears. If some or all lines are not grey, is black, a message appears, "There are lines which have not been injected. Continue?", click **Yes** to clear the worklist. (You will encounter this if a run had been interrupted and the worklist needs to be reimported. See VII.M.)
3. Insert the diskette.
4. Click **New Worklist**. There are six panels for the **Worklist Wizard / Worklist Creation Process**. The first one is the **Select data for worklist creation** panel.

NOTE: When the **Back** button is accessible, changes can be made to previous panels. The **Cancel** will close the worklist Wizard and

nothing is saved, the process must be started from the beginning. The **Finish** button will show the created worklist in its final form. No further changes can be made.

5. Click on **Import Punch File** from the **Available types of Worklist Sources** box. Click **Add** to move it to the **Sources to Use in this Worklist** box. To move a worklist source back, click on the source and the **Delete** button. Run Template, Run date, and Analyst box are also listed on this panel.
6. Enter the name/initials of the analyst, this can be up to three characters. This is a mandatory entry. If you do not enter your name, an error message appears in the **New Worklist** window,

Please enter the Analyst Name

Click **OK** and enter the name/initials of the analyst.

7. Click **Next**. The **Select Instruments for use** panel appears. Click a box to select or uncheck to deselect. Select each **Instrument #, Run Sys Begin, and Run Sys End**.
8. Click **Next**. The **Sample Source # 1: Punch File** panel appears.
9. Click **Browse** to see all the worklist files on the diskette. Worklist files are listed as yymmddrun#.ini. Highlight the correct file. Click **Open**. The selected worklist file appears in the **Select Punch file path** box.
10. Click **Next**. The **Select patient trays for run** panel appears. The patient trays for the run are listed. Select or deselect patient trays for the run by checking or removing the check from the appropriate box. The system trays are listed at the bottom.
11. Click **Next**. The **Instrument assignment** panel appears. This panel is used to confirm assignment of trays to instrument. This panel shows the

following information: Instrument #, Remaining tests (# of injections remaining on the column), Tray Cal and Pat Trays. Use the drop-down menu to change a tray's assignment to another instrument or change the order it is run.

12. Click **Next**. The **Worklist Preview** panel appears. The Instrument ID # is listed in Instrument Name box. Preview the trays with color-coded wells. The Instrument Name box has a drop-down list to select another instrument and its corresponding trays on the run.

13. Click on any tray to preview its worklist. A **Tray Preview** panel appears. The tray position and tray ID are listed with the Well Positions, Sample Type, Sample ID, Run, and Print columns.

Chromatograms are printed according to the following default setup:

Wells 3 – 8 followed by wells 85 - 87 for System Tray samples

All TQCs, well 16 or 1, 49, 96 or position of the last TQC

All PQCs

All CCs (carryover control), well 95 or the well before the last TQC

The **Deselect All** button below the Print column will clear all the boxes.

The **Restore Defaults** button will select boxes as setup in the

Instrument Worklist Settings. The default settings need a security access password to change.

14. Use the **Tray Preview** panel to create a partial worklist. Samples on each tray are selected by default according to the worklist imported. Delete samples by removing the check from the corresponding boxes in the **Run** column. There is also a **Deselect All** button below the **Run** column that will clear all the boxes. Then create the partial worklist by checking the boxes of samples to be run.
15. Verify selections to this panel are correct. Click the **Back** button to make any final changes to the previous panels if needed. When the **Finish**

button is clicked, further changes cannot be made. A new worklist will have to be created. Click **Save**.

16. Click **Finish**. The Worklist Wizard will close and the worklist appears on the screen. Scroll down the screen to verify the worklist is correct. If changes need to be made, the worklist has to be cleared and a new one created.

17. Repeat steps 4 through 16 to create another worklist on any available instrument.

F. Create a Manual Worklist, Backup Procedure , if worklist cannot be imported.

Click next to create worklist manually on the **worklist creation process** Sample source #2 : **Automatic Sample ID generation**. A complete worklist can be generated if the first and last accession ID's , accession year, Julian date and check digits are entered in the appropriate fields. **Click next** and the worklist appears on the next screen. The worklist is based on the same template found on the diskettes and can accommodate up to 349 patient samples.

For Barcode samples, manual entry can be done on sample source #2: **Manual Entry** screen. Enter tray ID #. Enter the sample IDs into the table. If the initial wells are used for non patient samples as defined in the template, navigate to the first patient sample ID using the Enter key. The complete sample ID will automatically appear and a new line will be added for the next sample. Repeat Until all sample Ids have been entered. Click the next tray button to start a new tray when multiple trays are necessary. **Click Next** and the worklist is displayed on the next screen. Follow steps 10-14 of worklist creation process described for importing worklist.

NOTE: 1. A given run with a manually created worklist can include only specimens barcoded with single site code. To make the run, change the **Lab Number** to the site for the specimens. A run with an

imported worklist can have specimens from any site.

2. Set up a separate run for each site code with the correct **Lab Number**.
3. For a given run with a manually created worklist, to handle different calendar years for specimens in the run, enter specimens from one year as a sequence with the **Accession Year** and specimens from a different year as a separate sequence with the **AccessionYear**. A run with an imported worklist can have specimens from any year.
4. All runs are automatically incremented.

G. Load Plates

1. Remove the evaporation cover, be careful and do not hit the sample probe or its protective shield.
2. Wipe any excess liquid from condensation. The NAS temperature control is 9 – 13 degree Celsius. Monitor the temperature on the bottom of screen. If systems are set up for automatic warm-up and not used each day, there may be condensation.
3. Load the Calib Tray in the Cal position of each NAS.
4. Load sample trays on each instrument as assigned in the **Worklist Map**. Trays are loaded into the NAS from right to left, sample trays in positions A to C.
5. Place the evaporation covers over the trays

H. Set up Printing

Printing is set up for you when the systems were installed. Positions which require printing are
Wells 3 – 8 for System Begin samples

All TQCs, well 16 or 1, 49, 96 or position of the last TQC
All PQCs
All CCs (carryover control), well 95 or the well before the last TQC
Wells 85 – 87 for System End samples

It requires a password, which is not provided, to change what samples are printed.

I. Analysis

1. Start the run. Select a system. The system must be in the **Ready** mode to start the run. Click **Run/Worklist/Start/Start**. The message, “Instrument started-Running state”, appears. Click **Close** when message is off the screen. Repeat with each system used in the run. The accession numbers on the worklist turn grey after completion of sampling and flushing.

NOTE: Worklist shows (Instrument) #, Tray #, Type (of sample), and Sample ID. Tray # is the position on the NAS.

Click **View Graphs** to see the chromatograms for the 3 systems. To view chromatograms from a single system, select the instrument by doubling clicking on the instrument icon, click **Run/Graph**.

2. Review results/chromatograms for the **System Tray** samples from the Calib Tray, pos 3 – 8, from the printout to verify that all parameters are within limits set by GDL. after sampling cup 8, the probe is flushed, system remains in **Running** mode, and **Start/Stop** is inaccessible with the red light on. The sample probe goes to cup 16 or cup 1, the leading TQC of the 1st Pat tray after the Calib Tray for the system (most often it is the Pat

tray in position A of NAS), and samples the TQC. The system then holds the sample in the injection loop.

Be sure the 1st Pat tray for the system is loaded before cup 8 is sampled. When the chromatogram is completed for cup 8, an alarm sounds. The screen shows: System tray is completed Please go to worklist screen to continue run . OK Click **OK**.

The system then pauses for up to 1 hour. On the screen, the status of the system is **Paused**. You must review the results within the hour and resumed testing, otherwise, the system will automatically stop the run. The sample held in the injection loop is discarded to waste. You cannot restart the run without importing the worklist again.

NOTE: During the **Pause** and **Ready** mode, the pumps are not pumping and there is no worry about stripping the column.

If **System Tray** samples failed to meet limits set by GDL and a system cannot be used,

- a. Determine which Pat trays were assigned to the system that failed to meet limits.
- b. Clear the worklist for each system and reimport the worklist and reassign all Pat trays to the two remaining systems. This assumes that during the pause, the run had not yet started for any system. If the run had start for any system,
 - 1) Determine which Pat trays were assigned to the system that failed to meet limits.
 - 2) Let a system complete testing all its Pat trays and System End.
 - 3) Clear the worklist for the system if needed.
 - 4) Reimport the worklist.
 - 5) Deselect Pat trays that are assigned to the system that is still running or has completed running.

NOTE: This function is not available if the worklist is manually created.

- 6) Select the system.

- 7) Assign the Pat tray to this system. Run # increments and you do not have to run System Begin samples.
3. To proceed to test the sample trays, click **Run/Worklist/Start/Continue/Close**. The run continues by injecting the tray control held in the injection loop that was sampled from cup 16 or 1 of the patient tray in position A. The sample probe then starts sampling from cup 17 or 2. Therefore, cup 16 or 1 is no longer sampled twice and every microtiter plate can be used for a second sampling if needed. The run continues until all sample trays and the System End samples are tested. Run is completed when you see well # 87 as the last chromatogram on the View Graph screen.
NOTE: After sampling and injecting the last TQC after the last patient, the probe goes to cup 85 of the Calib Tray to sample and inject. System then pauses between trays. After about a 10 minute pause, the probe then samples and injects cup 86 and 87.
 4. To pause the run, click **Stop/Pause/Close**. The system finishes the sampling, injection, and flushing steps with system status in **Running** mode. There is no result for this injection. The system then pauses, system status is **Paused**, and the yellow light is on. To restart, click **Start/Continue/Close**. The system starts the run by going back to the last well sampled. The sample probe begins by sampling the last well it sampled so that the well, when the pause occurred, is sampled twice. This result is valid. The analysis proceeds.
NOTE: Going from **Pause** to **Continue**, “Exit Pause state operation-Gradient” is on the upper left of the screen.
 5. To stop a run, click **Start/Stop/Close**. Once stopped, the worklist cannot be restarted. You may have to repunch, reimport, etc.
 6. Evaluate hemoglobin patterns at the completion of the run. When all accession numbers on the worklist turned from black to grey. For each worklist, a Summary Report NeoHb is printed automatically at the end of the run. The report lists Tray Type, Well, Sample Type, Accession

Number, %F1, %F, %A, %E, %D, %S, %C, %Other (name of window-area%), and Hb Pattern. 1-.4 on the pattern report means a peak in the Other 1 window at 0.4 area %. To manually print the summary report click **Data/View Run**. Select the run and click **Print**. Examine the printout to assure that the last sample in the run has been analyzed.

7. Score the trays/results.
 - a. Click **Data/View Run**.
 - b. Select each tray and click the down arrow under **Rating** to score **Release, Hold, or Prevent** following rules set by GDL. Continue until every tray is scored.
 - c. Highlight a tray to see a listing of the individual results on that tray. The information for each result are Injection #, Type (of sample), Sample ID, Rating, Pattern, each Hb peak as area %. Highlight a sample and click **View Sample** to see the chromatogram, each Hb peak with Height, RT, Area, and Area %.
 - d. Score an individual result by using the down arrow under Rating to score **Release, Hold, or Prevent**.
 - e. Score **Prevent** for individual results if
 - 1) The sample is tested for BIO or TRA.
 - 2) The samples are not for newborn screening.
 - f. Score **Hold** for an individual result if you want additional review by GDL QA.
8. Send all data to GDL QA. On GDM 3.0 Data screen, System Begin and System End trays are treated as one plate.

- a. Highlight any tray from the worklist (or run #), e.g., System tray or Pat. tray for instrument #1, and the entire worklist (or run #) will be transmitted. Click **Transmit**.
 - b. Repeat for each worklist. Each worklist (run #) must be transmitted separately.
 - c. Click **Send/Close**. As each tray is sent, a **Yes** appears in the **Sent** column.
9. Fill out information on the Hemoglobinopathies Checklist for each run. FAX checklist to GDL after all data is transferred. If the worklist was manually created (Section VII.F.), also FAX the TRA worklist to allow GDL to verify that the hemoglobins were run in the order punched.
- J. Hb Patterns of F ONLY, NO PEAK, LOW AREA, HIGH AREA, or FAST and F1 >50%
1. F only
 - a. Review the Summary Report NeoHb for F only pattern.
 - b. Repeat testing for Hb no later than the next day by repunching the specimen.
 2. NO PEAK, LOW AREA, or HIGH AREA

When a result printed in the Pattern Report is NO PEAK, LOW AREA, or HIGH AREA, it is automatically prevented by the system and you must retest by the next day. Follow these instructions to determine if an error occurred. If an inconsistency is found, take appropriate corrective action to assure that only correct results are reported.

- a. Compare the accession number from the NeoGo worklist with the accession number on the Hb pattern report to assure that they agree.
 - b. Assure that positional controls are in the correct well for all trays.
 - c. Check grandmother, mother, and daughter plates for all analytes to determine any source of error. Carefully check the Hb microplate for liquid level in the affected well, and for color of the liquid relative to the other wells. If the pattern is LOW AREA, check the grandmother tray for the number of spots in the well. If the number is not 1, check other wells for 2 spots.
 - d. Check the specimen card to determine if a single 6 mm disk has been punched. Record relevant observations made regarding microplate and specimen cards.
 - e. Prevent the results for HB , BIO , Transferase , and repeat the next day.
 - f. Declare the specimen inadequate if the pattern is confirmed on repeat testing.
 - g. Maintain a tabulation of all repeats due to NO PEAK or LOW or HIGH AREA. The tabulation will indicate if procedures for judging adequacy need to be reviewed with the accession technician, or if maintenance is needed on the instrumentation. Instruments to be examined are the Puncher, TomTec, and the HPLC system (if only Hb results are discrepant).
3. FAST and F1 >50%
- Result is held when FAST and F1>50% and may have a Hb pattern of FAB. Do not retest unless instructed by GDL.

K Shutdown procedures

1. Check the **Well #** on the screen. When the system has completed testing all samples, **Well #** is 87.
2. Check status of each system. If your systems are setup for automatic warm-up, it is automatic shutdown. There is nothing you need to do other than unload the microtiter plates.
3. Proceed to Preparation for Analysis and begin your next runs the next day.
4. To shutdown your systems if they are not setup for automatic warm-up, there is also nothing for you to do except unload the microtiter plates. The pumps will stop on its own and the system will go to the **Inactive** mode 1 hour after completion or run. Next morning you systems are in the **Inactive** mode and you need to start the warm-up procedure.

L. Repeat Testing

For repeat testing, eluates from the round deep well mother tray can be used for dilution and distribution into a new microtiter tray. Cover the tray, refrigerate, and test by the next day if repeat testing is needed when the cause for the out of control run is not due to punching, elution, or filtration. Stability studies at GDL show that the diluted eluates in microtiter trays are stable for 48 hours when refrigerated.

All microtiter trays of diluted eluates can be sampled a second time (unless you click **Stop/Pause/Close** during the run in which case one well was sampled twice. See VII.I.4.)

M. Interrupted Run

A run can be interrupted by a power failure or other problem that shuts down all

three column systems at the same time, or one of the three systems alone may come to a halt (*e.g.*, lock up) while the remaining two systems continue to function.

1. Restore power to each system affected. Click **Maintain/Instruments/Return to Ready State** for each system.
 2. Determine where each system stopped by viewing the worklist for the run. Samples processed will be grey. Also click **Data/View Run** to find completed tray.
 3. Click **Run/Worklist/Clear Worklist/Clear**. Click **Yes** when the prompt appears, "There are lines which have not been injected. Continue?" Re-import the worklist and select only the trays which have samples that need to be tested
 4. Deselect by unchecking the tray numbers for all trays which have been completely analyzed..
NOTE: This option to deselect trays is not available when the worklist is manually created and the system is interrupted. All samples must be retested.
 5. Select the systems for the run and assign the Pat trays to them.
 6. Ensure **System trays** are selected for each system. Even though this is not the first run of the day, after an interruption, run **System Tray** to verify satisfactory performance of each system.
 7. Click **Create Partial Worklist** for the trays which have been partially analyzed. If interruption happens when sampling wells 1-49, re-run the entire tray. If interruption happens when sampling wells 50-96 create partial worklist by checking samples from 49-96 on the worklist. Just as a completed tray is acceptable without System End samples, the first half of a tray with bracketing TQC is also acceptable.
 8. Restart the run as previously described. Run # is incremented by 1.
- N. Backup daily. Ensure that all systems are in the **Ready** or **Inactive** mode.
1. Click **Setup/Configuration** and place CD in CD-ROM.
 2. Click **Database Backup**.

3. Select **Backup** and clear. CD is formatted automatically when it is on the same screen.
 4. Click **OK**. Database will be backed up. Message on the screen is, "Please wait. Database backup in progress."
 5. A completion screen will be displayed following successful backup; click **Finish** button to close the screen.
 6. To restore old data, refer to Bio Rad operational manual for GDM 3.0.
- O. View chromatograms from previous runs
1. Verify that the system used for testing is in the **Ready** mode.
 2. Click **Data/View Run**. The number of runs for the system is listed as
Number of Runs.
 3. Highlight, by test date, the run containing chromatograms you need to view. Each run has the System Tray, and Pat trays list individually by tray. Depending on the chromatogram you wish to view, you need to click on the correct individual tray by date. Once selected, the number of samples in the tray is listed as **Number of Samples**.
 4. View the part of the screen containing **Inj #, Type** (of sample), **Sample ID, Rating, Pattern, Fast, F1**, and all other HB pk with Area %. Included in this table are all the samples from the selected tray.
 5. Highlight the sample and click **View Sample**. The data and chromatogram for the sample appears on the screen. On the screen is also the worklist for the tray and you can select any sample to view its chromatogram.
 6. Click **Expand** if you want a larger view of the chromatogram.
 7. Click the **Print** icon. Select to print **Current sample, All Samples in View**, or samples **From Inj# to Inj#**.
- P. Maintenance
1. General
 - a. To protect and prolong their useful life of CDs, observe the following precautions.
 - 1) Handle CD only by edge to prevent fingerprints and

smears on the surface. Store disc in protective case.

- 2) Do not leave CD in direct sunlight or in a hot, humid environment.
- 3) Keep CD away from heat sources, such as warm air ducts or lamps.
- 4) Do not stick adhesive label on CD, use only a felt tip permanent pen when labeling. Write only on the printed area or on the clear inner diameter of the disc.
- 5) To clean the CD, use a soft, lint-free cloth to remove spots, dust or fingerprints. Always wipe from the center to the outside edge and never wipe in a circular motion.
- 6) Store the CDs in a cool, dust-free, non-magnetic environment.

2. Weekly

Record all maintenance activities on the Weekly Preventive Maintenance chart. See Attachment 4.

- a. Clean the wash/flush station.
 - 1) Move the sample probe away from the wash/flush station by clicking **Maintain/Go to Base**.
 - 2) Swab the wash/flush station with bleach and rinse with distilled water.
- b. Clean the outside of the sample probe.
 - 1) Turn power off to the NAS.
 - 2) Unscrew the tubing from the sample probe and push the probe up to remove. Insert the cleaning wire in the probe to remove any dried blood from the samples deposited

inside the probe. Wash under the distilal water tap and wipe the outside of the sample probe with a Kimwipe soaked with bleach. Remember to wear surgical gloves when carrying out this procedure and take special attention not to touch the tip of the probe.

3) Rinse well with distilled water, dry and screw it back on.

c. Backup files and clear the database. This operation must be performed at the end of the day after all runs are completed or at the beginning of the day prior to the start of any runs.

1) Use a new CD each week. Write the date when files are backed up onto the CD.

2) Click **Setup/Configuration** screen.

3) Click **Database Backup** button. This function can be used to back up the GDM database and method files to the hard drive or a CD-R.

4) Place a new CD in CD-ROM. A new CD is formatted when the Back up to CD Writer box is checked on the database backup screen as described later.

5) Select the **Backup and Clear** box. Click OK.

Backup and Clear removes data from the database.

The location and the name of the database file will be

D:\GDM3.0\BackupDB\ddmmyyyy-hh-mm-

GDM.zbk. The GDM 3.0 database is limited to

approximately 14,000 injections. When the number of injections for the run exceeds the maximum allowed,

the run is completed and GDM stops running. A

message is displayed, prompting the user to perform database **Backup and Clear**.

- 6) If the database is being saved to a CD, leave the **Backup To CD writer** check box selected and insert the CD into the CD-RW drive. If the CD is unformatted, the dialog box on the screen displays an option box **Take No Action**, click **O.K.** It displays **CD Writing Wizard** screen. Click **NEXT**. CD is formatted and Data is backed up on the CD.
 - 7) Keep the CD in your laboratory for the length of time to meet CLIA requirements.
 - 8) Call GDL if your laboratory needs to load data files from the CD to the hard drive.
- d. Shut down the computer. Close the GDM software. Click **Start/Shutdown**. Select **Shut down the computer**. Click Yes. Turn power off when the message, “It is now safe to turn off your computer”, appears. Wait 10 – 15 seconds turn power on.
3. Monthly
- Record all maintenance activities on the Monthly /Periodic Preventive Maintenance chart. See Attachment 5.
- a. Perform bleaching procedure once a month on each system or more frequently as needed.
 - 1) Remove the analytical column and install the dummy column. Click **Setup/Test/Cartridges** and enter for the

dummy column Dummy as **Lot #**, 2000 as **Inj Limit**, and Yes for **In Use**.

NOTE: Running this procedure with the analytical column instead of dummy column will destroy the analytical column.

- 2) Fill wells 16 to 20 on the microtiter tray with 5% sodium hypochlorite solution (undiluted household bleach).
- 3) Fill 20 subsequent wells with distilled water.
- 4) Place the microtiter plate in position A of the NAS.
- 5) Use the disk containing the special worklist named “Decon” and import the worklist. Deselect **System Tray**.
- 6) Reinstall the analytical column when completed.

b. Clean exterior/interior surfaces.

- 1) Exterior surface cleaning:

Use a cloth or sponge dampened with water to wipe the exterior surface of the system. Do not use abrasive cleaners. If required, use a mild soap solution diluted with water to clean the surface, then wipe with a damp cloth or sponge to remove any soap residue.

- 2) Interior surface cleaning

Wipe up any fluid using a soft disposable towel or tissue. Be sure to clean fluid from the lower interior surface. Tighten any leaking connections. Clean the interior base plate of the system by wiping it with a soft cloth or sponge dampened with water.

- 3) Clean PC printer with damp cloth to remove dust.

c. Apricot GTPS-96

Apricot is used for the dilution and distribution of the eluate used for the hemoglobin testing. The pipet for each well must aspirate a sample volume of $38\mu\text{L} \pm 1\mu\text{L}$ and must dilute with a volume of $176\mu\text{L} \pm 2\mu\text{L}$. To monitor the accuracy and precision of apricot, see page 30 of the Specimen Handling, the Processing of Blood Spots Using Specimen Gate for Newborn Screening. Tracking No. CN 003, Version 7.0.

4. Periodic

Bio-Rad will provide periodic preventive maintenance and will check/change such things as tubing and pump seals on the NHS, alignment of sample probe, lamp, etc

Q. Troubleshooting

1. To verify performance of a system after troubleshooting, if needed.
Run the System tray samples.
 - a. Import any worklist or generate a “dummy” manual worklist.
 - b. Set up a Calib Tray with System Begin samples.
 - c. Start the run, run only wells 2 – 8, then stop the run. Run primer if needed.
 - d. Examine the results. Take corrective action if needed, otherwise Proceed with the analysis.
2. See also Attachment 9, Troubleshooting Common assay problems for Hb

Contact Bio-Rad if needed, see Attachment 6 for instructions.

VIII. Calculations

The HPLC screening method automatically quantifies the concentration of each

hemoglobin variant peak. Area percent of each hemoglobin is used to derive automatically the Hb pattern for each newborn. Hemoglobins are listed in decreasing order by area percent. No further calculations are needed.

IX. Reporting Results

Before results are reported, results are validated using a quality control program.

A. Quality Control Program

The quality control program is designed to

- Monitor the performance of the HPLC systems.
- Monitor the day-to-day performance of the method including the punch and elution steps.
- Confirm that the sample trays were processed in the correct sequence.

Three types of quality control samples are used for this assay to meet the intended goals. The three types are system controls, tray controls, and positional controls.

The tray controls CT and CP are blood spot controls prepared and provided By GDL. The system control is prepared and provided by Bio-Rad as part of its reagent system. Positional control is a water cup.

The chromatograms and the area/percent reports are printed for each sample.

Use the printout to determine that quality control parameters for all trays are within action limits set by GDL. See Attachment 1, Quality Control Rules, Attachments 2, Quality Control Action Chart, and Attachment 3, Action Limits.

B. Reporting Results

1. Transmit data from NAPS labs to GDL QA system after reviewed and

released by the supervisor. See Section VII.I.7.- 8. Contact Bio-Rad if transmission is not completed successfully.

2. FAX the Hemoglobinopathies Checklist to GDL. Also FAX the TRA worklist if the Hb worklist was manually created. Checklists are due at GDL before 9 AM the following morning.

X. Procedure Notes

- A. Each new lot of resin will come with a CD that contains the new gradient. On the start up date of the new resin, load the new gradient onto your systems. Go to **Setup/Test/Update Kit**. Select E drive. The one file on the CD will open, click OK to load.
- B. This additional step must be performed when a new lot of buffer is first used. After moving the lines into the bottles of the new lot, click **Setup/Test/Cartridges/Start System Flush**. This takes about 2 – 3 minutes.

XI. Limitations of Procedure

Accurate recognition of hemoglobins A, F, E, D, S and C is dependent upon the method used. Newborn screening results may differ from clinical follow up results because other variants elute at retention times for Hb S, E, D, and C. In the first four years of screening using the automated HPLC method where 2.5 million newborns were tested, GDL has been notified of 12 instances in which diagnostic follow up results did not agree with the newborn screening hemoglobin pattern. HPLC screening method has the following limitations:

- A. Hb variant elutes on HPLC as Hb S. Incidence 1/10,000 (1% of FAS pattern).
- B. Similarly the newborn pattern is FAE but the follow up pattern is FAV. Hb variant elutes on HPLC as Hb E.
- C. Hb G has been reported in newborn screening as Hb E, Hb D.

XII. References

Wilson, J., Headlee, M.E., Huisman, T.H.J., "A new high performance liquid chromatographic procedure for the separation and quantitation of various hemoglobin variants in adults and newborn babies", Journal of Laboratory and Clinical Medicine, 102, 174-186, 1983.

Eastman, J.W., Wong, R., Liao, C.L., Morales, D.R., " Automated HPLC screening of newborns for sickle cell anemia and other hemoglobinopathies", Clinical Chemistry, 42, 704-710, 1996.

Attachment 1

QUALITY CONTROL RULES

Each chromatographic column system is treated as an independent analytical system. Quality control rules and corrective actions apply to each column system separately. The quality control parameters are (a) the F- and S-hemoglobin peak values determined on the Calib and newborn trays, (b) positional controls, (c) the hemoglobin patterns, and (d) pump pressure tracings.

Two result values accompany the measurement of each chromatographic peak: amplitude and time. The amplitude is a measure of concentration and may be in "height", "area", or "area percent" units. "Area percent" will not be used. The time is the retention time in minutes.

Quality control readings are taken from the printout that accompany the HPLC analysis of each calibrator or quality control sample. The hemoglobin pattern is taken from the "NeoHb Summary Report" that is printed out at the end of each run.

The uses of the system controls on the Calib Tray and the tray controls are charted in Table 1, Quality Control Action Chart.

Laboratory data collected by GDL and NAPS laboratories are used to set the action limits in Table 2, Action Limits. The retention times given are those that were applied to a particular lot of cation exchange resin. For the limits that apply to the current lot of resin, refer to the posted action limits.

(Note 1: In printing out the AREA/PERCENT REPORT, the HPLC controller drops off the trailing digits of each number. Nevertheless, the trailing digits are used to determine the position of a hemoglobin peak in a particular window. For example, an S-peak with a retention time of 1.245 minutes is printed out as hemoglobin "OTHER 4" with a retention time of 1.245 minutes. In order to score quality control results, the retention time is to be

considered beyond acceptable limits if it is reported with the incorrect name. For example, a hemoglobin S peak printed out as OTHER 4 is beyond limits, even though the retention time is 1.24, which is on the edge of the limits.)

(Note 2: If a hemoglobin peak does not appear in the correct window of retention times, then the analyst must check adjacent windows in order to score the correctness of the area.

For example, if S-hemoglobin is reported with a retention time of 1.25 minutes, the peak fails to pass the test of retention time. The peak appears as OTHER (4). In this case the analyst will read the area value for OTHER (4) to see if it falls within the action limits for the S-hemoglobin area.)

I. CORRECTIVE ACTION

Whenever an out-of-control tray is observed, the supervisor must immediately make a determination of the magnitude of the error. If a gross error has occurred the HPLC run must be terminated and corrective action taken. If a gross error has not occurred then the HPLC run should be allowed to continue.

A. Gross Error Observed

A gross error is one that is so severe that the chromatogram does not look right with casual inspection. For example, the tray control may show only one smeared-out peak, or the pump pressure may show a discontinuity.

If it is clear to the supervisor that a gross error has occurred, the cause of the error must be identified and corrected before the HPLC run can be continued. In most cases, the cause of the error will be an obvious failure to follow protocol. For example, one of the pumps may be turned off, or a bottle of elution buffer may have gone dry, or there may be buffer leaking from one of the joints at the end of the cation exchange cartridge. These gross errors, usually due to failures to follow protocol, are so severe that an HPLC separation cannot be attempted.

B. Gross Error Not Observed

If the supervisor determines that no gross error has occurred, then the HPLC run should be continued. For example, if the retention time of the S-hemoglobin peak has shifted beyond the \pm limits, but the overall appearance of the chromatogram is retained, the HPLC run should not be terminated.

GDL will review all runs marked by the supervisor as "H" (see below). If a repeat analysis is needed, GDL will telephone the laboratory to request that a repeat be carried out or that the specimens be forwarded to GDL for analysis. In most cases only a few representative samples and positives may be repeated.

C. Identify Trays for Review

Trays that have been flagged by the quality control rules must be scored as **“H”** or **“P”**. These trays are not automatically subjected to a repeat analysis, unless a gross error has occurred.

D. Repeat Analyses

1. Use same microtiter plate.

If no error has occurred during punching, elution, and distribution of the shared eluate, the same plate may be used for a second set of HPLC analyses. There is enough liquid in the microtiter plate for two HPLC runs.

2. Prepare a new eluate. In some cases a new blood spot must be used.

Punch the specimen using repeat mode per Specimen Handling protocol.

II. QUALITY CONTROL CHARTS

Use quality control charts to plot the following parameters:

- A. Area counts, hemoglobin F, linearity standard #3, located at position 6 in the Calib Tray.
- B. Retention time, hemoglobins F and S, System Control I, located at positions 7 and 86 in the Calib Tray.
- C. Retention time and area counts, hemoglobin S, tray control, located at positions 1 or 16, 49, and 96 on each tray of newborn specimens.

III. CALIBRATION

The configuration of the Calib Tray is given in the protocol. The limits are applied to every Calib Tray. Apply the quality control criteria in Tables 1 and 2 as described in the following:

- A. Baseline

If there is any compound listed on the AREA/PERCENT REPORT, the Height must be less than 6000 counts.

- B. Sensitivity

The area of the F peak at position 6 must be within the limits shown on Table 2.

- C. Hemoglobin separation

The F and S peaks at position 7 must yield values that fall within limits given for the Retention Time.

D. Gross Error

The following conditions constitute gross errors. An HPLC system cannot be used if any of these observations is made on the Calib Tray.

1. Area, hemoglobin F, Cup #6, area < 1,300,000 counts.
2. Retention Time (RT), hemoglobin S, Cup #7 $RT \leq 1.15$ or $RT \geq 1.25$. These limits apply to a particular lot of cation exchange resin. For the limits that apply to the current lot of resin, refer to the posted current limits for gross error.
3. In cups 4-6, the calibrators (FA), and in cups 7 and 8, the system controls (FAES and FADC), do not show well resolved bell-shaped curves for every hemoglobin.
4. The quotient of HEIGHT divided by AREA is less than .22 for hemoglobin F determined at position 8 (FADC control).

If these gross errors are observed you must take corrective action and repeat.

IV. NEWBORN TRAYS

The configuration of the sample tray is given in the protocol.

A. Tray Controls

The S peaks for positions 16, 49, and 96 must yield values that fall within the action limits for the following two parameters: Retention Time, Area Counts.

B. Positional Controls

If there is any compound shown on the AREA/PERCENT report, the Height must be less than 6000 counts. If there is a Pat tray mix-up, the analysis of the affected trays must be repeated.

V. POSITIONS 86 OF CALLIB TRAY

The F and S peaks for System Control I must yield values that fall within limits for the Retention Time.

VI. PUMP PRESSURE TRACINGS

Pump pressure tracings must show a typical recording of the voltage at about .26 volts level on the chromatographs printed out in the process of a run. If an irregular pattern is noted, e.g., a spike or a drift up or down, observe the following rules.

A. Gross Error

A major drop or rise in voltage or a definite drift constitutes a gross error if it is observed on the Calib Tray or at position 1 of a newborn specimen tray. Stop the analysis and take corrective action before proceeding with the analyses of newborn specimens.

B. Not a Gross Error

If a pattern of spikes or drifts are noted on the printout for the positional control, at position 49 of a newborn specimen tray, or at position 86 or 87, GDL will review the chromatograms and determine if corrective action is needed. Flag the tray "H", and enter a comment on the checklist ("review pressure tracing on tray __").

VII. CHROMATOGRAMS and HEMOGLOBIN PATTERNS

The analyst should spot check the appearance of chromatograms on the CRT monitor. If a gross error occurs, appropriate corrective action should be taken. At the end of the run, review the patterns on the NeoHb Summary Report. Following samples must have the following patterns:

Calibrators: FA

System Control I: FAES

System Control II: FACD or FACDE

Newborn Specimens: FA, with few exceptions (presumptive positives)

As a rule, the number of presumptive positive newborn test results should not exceed about ten per day. Do not list FA(1) as a presumptive positive.

VIII. HEMOGLOBINOPATHIES CHECKLIST

The hemoglobinopathies checklist, Attachment 7, is used to record the results of the controls. Enter the observed reading in the space provide. Do not enter the last three digits of area readings. If a parameter exceeds acceptable limits, the analyst will mark the out-of-control reading with an asterisk (*). Also, in the space provided enter "R" if the tray is satisfactory, enter "H" if the tray is flagged for GDL review, or enter "P" for trays to be repeated. If a pattern is NOT DETERMINED, then record the parenthetical code, *e.g.*, >3 PEAKS, in the space designated for the pattern. Also, list specimens that are printed with the warning of "LOW AREA" or "HIGH AREA". Transmit the Hemoglobinopathies Checklist to GDL via FAX. FAX transmissions should be made before 9 am.

Attachment 2

TABLE 1
 QUALITY CONTROL ACTION CHART

QUALITY CONTROL RESULTS				ACTIONS AT THE SCREENING LABORATORY
ITEM	SYSTEM CONTROLS	TRAY CONTROLS	POSITIONAL CONTROLS	
1	IN	IN	IN	NO ACTION
2	OUT	IN	IN	I. Gross error A. Identify and correct all failures to follow protocol. B. Repeat the out-of-control tray II. Not a gross error A. Continue the assay B. At the end of the run, flag the tray as "H".
3	IN	OUT	IN	I. Gross error A. Identify and correct all failures to follow protocol. B. Repeat the out-of-control trays. C. Flag out-of-control trays as "P" II. Not a gross error A. Continue the assay. B. At the end of the run, flag the tray as "H".
4	IN	IN	OUT	I. No tray or sample mix-up A. Continue assays. B. At the end of the run, flag the tray as "H". II. Tray or sample mix-up A. Flag the tray "P". B. Repeat the analysis of

				affected trays on next run
--	--	--	--	----------------------------

Attachment 3

TABLE 2 (November 2001)

ACTION LIMITS

Tray Control, Lot # ____

I. GROSS ERRORS

Retention Time and Area				
Tray	Cup	Hemoglobin	Retention Time (min)	Area (counts)
Calib Tray	6	F		<1,300,000
Calib Tray	7	S	≤ 1.15 or ≥ 1.25.	

II. FLAGS

Retention Time and Area				
Tray	Cup	Hemoglobin	Retention Time (min)	Area (counts)
Calib Tray	6	F		1,560,000 – 2,340,000
Calib Tray	7	F	0.58- 0.72	...
Calib Tray	86			
Calib Tray	7	S	1.16–1.24	...

Calib Tray	86			
01,	16,49,96	S	1.16–1.24	refer to current limits
02,03,04	1,49,96	S	1.16 – 1.24	Refer to current limits

Height		
Tray	Cup	Height
Calib Tray	3	
01	25	<6000 Counts
02	26	
03	27	
04.	28	
Calib Tray	85	

Attachment 4

WEEKLY PREVENTIVE MAINTENANCE RECORD

Instructions: Perform weekly maintenance per protocol. When completed, initial the appropriate week.

Year _____

Date																						
Item																						
Clean Wash/Flush Station																						
Clean outside of Sample Probe																						
Backup and clear database																						
Shut down computer																						

Attachment 5

MONTHLY PREVENTIVE MAINTENANCE RECORD

Instructions: Perform monthly maintenance per protocol. When completed, initial the appropriate week.

Year _____

Month	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Item												
Perform bleaching procedure												
Clean exterior/interior surfaces												
Quadra A & P												

Attachment 6

Instructions for Bio-Rad Service

1. Call the Instrument Service Department (ISD) in Hercules, CA at 1(888) 204-6164.
2. The ISD is staffed by Field Service Representatives. The group is available to answer inquiries pertaining to the operation of the HPLC-based Hemoglobin Variants analyzer according to the time schedule below. The ISD is also the dispatch center for our Field Service Representatives. If it is decided that a Field Representative is needed to service your equipment, the ISD will do so. The representative will contact you to schedule the service call.
3. Provide the following information when you place your call to the ISD and whenever you leave a message with the operator:
 - a. Your name.
 - b. Instrument Model and Serial Number.
 - c. Your institution name, address and phone number. Identify that you are part of the California Newborn Screening Program for hemoglobins.
 - d. For each instrument malfunction:
 - 1) Describe all symptoms of malfunction.
 - 2) Describe any steps that you have taken to correct malfunction (diagnostics performed, troubleshooting steps taken, etc.)
 - 3) Save all printed tapes and test data. Have available for discussion.
 - 4) Have the Operator's Manual available for reference.
 - 5) Use the telephone located nearest to the instrument whenever possible.

TIME SCHEDULE**Monday - Friday**

5:00 a.m. - 5:00 p.m. (PST) ISD available for troubleshooting.

5:00 p.m. - 5:00 a.m. (PST) Message will be taken and ISD will return your call.

Saturday-Sunday

Messages will be taken 24 hours a day over the weekend and ISD will return the call ASAP.

For handy reference, note the serial numbers in the spaces below:

INSTRUMENT

SERIAL NUMBER

Attachment 7

HEMOBLOBINOPATHIES CHECKLIST

Site Code: _____ Analyst: _____ Date: _____ Run: _____ of _____

Run reviewed by:

Name _____ Date _____

Transfer to Tape:

Time _____ Date _____ Start Transfer to GDL: Time _____ Date _____

VIII. List of Positives

Item	Accession Number	Pattern	Item	Accession Number	Pattern	Item	Accession Number	Pattern
1.			5.			9.		
2.			6.			10.		
3.			7.			11.		
4.			8.			12.		

System	Column Lot	Serial Number	Injection Counter	Temperature	Standard I	Lot # _____	Date of Recons _____
1	_____	_____	_____	_____	Standard II	Lot # _____	Date of Recons _____
2	_____	_____	_____	_____	Standard III	Lot # _____	Date of Recons _____
3	_____	_____	_____	_____	Control I	Lot # _____	Date of Recons _____
					Control II	Lot # _____	Date of Recons _____
Tray control Lot #	_____				Buffer A	Lot # _____	
Last specimen is in tray	_____ cup _____				Buffer B	Lot # _____	
					Primer	Lot # _____	

COMMENTS

Site Code: _____

Date: __/__/__

Run: ___ of _____

CALIBRATION

R	LIMITS		Sys 1						Sys2						Sys 3					
			START			END			START			END			START			END		
	H	Height/ Area	101 : R/H/P			111: R/H/P			102: R/H/P			112: R/H/P			103: R/H/P			113: R/H/P		
Blk	---	<6000	Cup	R	H/A	Cup	R	H/A	Cup	R	H/A	Cup	R	H	Cup	R	H/A	Cup	R	H
F	---	1,560,000 --- 2,340,000	3	-	<u>H</u>	1	-		3	-	<u>H</u>	1	-		3	-	<u>H</u>	1	-	
F	---		6	-	<u>A</u>	-	-	-	6	-	<u>A</u>	-	-	-	6	-	<u>A</u>	-	-	-
F	0.55- 0.69	---	7		-	86		-	7		-	86		-	7		-	86		-
S	1.15- 1.25	---	7		-	86		-	7		-	86		-	7		-	86		-

NEWBORN TRAYS

Cup:		NeoGo Frame Number	1		P	49		96		R/H/P
Parameter:	RT		Area	Height	RT	Area	RT	Area		
Limits:	1.15		--	<6000	1.15	--	1.15	--		
	--				--		--			
Tray	System									
1										
2										
3										
4										
5										

Attachment 8

Troubleshooting Common Assay Problems for Hemoglobin

Event	Cause	Action
Calibrator(s) outside limits	Poor reconstitution	Reconstitute per protocol
	Inaccurate pipette	Use a different pipette
	Bad Std due to inappropriate storage	Store per protocol
	Sample probe dirty on outside, dirty wash well	Clean outside probe and wash well with bleach and rinse with distilled water
	Bad column	Change column
	Incorrect temperature	Call Bio-Rad
Blank is high	Contaminated water	Determine source of contamination and correct, discard and use clean water
	Carryover from incorrectly working pumps	Call Bio-Rad
	Dirty pipette if on Sys Begin	Clean the end of pipette tip
	Sample probe dirty on outside, dirty wash well	Clean outside probe and wash well with bleach and rinse with distilled water
	Dirty system if for many water cups	Perform monthly bleach procedure
System control outside limits		
By %area	Poor reconstitution	Reconstitute per protocol
	Inaccurate pipette	Use a different pipette
	Bad SQC due to inappropriate storage	Store per protocol
	Sample probe dirty on outside, dirty wash well	Clean outside probe and wash well with bleach and rinse with distilled water
	Leak in injection valve	Look for drops on sample probe indicating a leak, call Bio-Rad
	Poor resolution	Check for leaks, crimped tubing, pumps not working, blockage in system, and call Bio-Rad
	By retention time	Improperly seated column
Leak in sys, pumps, injection valve, or		Identify source of leak if possible and call Bio-

	Autosampler	Rad
	Incorrect temperature	Call Bio-Rad
	Blockage in Autosampler	Perform bleaching procedure, call Bio-Rad if needed
	Crimp or blockage in tubing	Uncrimp if possible, call Bio-Rad
Tray control outside limits		
By % area	Poor elution with spot outside of liquid	Tap tray before elution to have spot in liquid and get rid of air bubbles
	Bad punch	Punch per protocol with head on entire spot
	Bad Quadra tip that did not dilute and dispense	Check tips before loading, check O rings on Quadra, always lubricate
	No spot in well for elution	Check square deep well tray for spot, use Zerostat when punching
	Sample probe dirty on outside, dirty wash well	Clean outside probe and wash well with bleach and rinse with distilled water
	Leak in injection valve	Look for drops on sample probe indicating a leak, call Bio-Rad
By retention times	Bad column	Change column
	Leak in pump tubing and fittings	Find leak if possible and call Bio-Rad
	Incorrect temperature	Call Bio-Rad
System has low/high bias		
Low bias	Leaks in system at cartridge holder, tubing and fittings	Find leak if possible, tighten fittings, call Bio-Rad
	Bad lamp	Call Bio-Rad
	Bad column	Change column
	Dirty flow cell	Perform bleaching procedure
	Blockage in Autosampler syringe, tubing, or injection valve	Perform bleaching procedure
High bias	Blockage in detector	Call Bio-Rad
	Bad lamp	Call Bio-Rad

	Dirty flow cell	Perform bleaching procedure
Repeats are inconsistent	Contaminated eluate or spot	Handle filter paper with gloves, place on clean surface, do not touch spots, cover plate after distribution, store plates in clean place
	2 patients on filter paper	If confirmed, call GDL
	Leaks in system at cartridge holder, tubing and fittings	Find leak if possible, tighten fittings, call Bio-Rad
	Unstable pump pressure	Check for crimps in tubing and leaks in system and call Bio-Rad, make sure reagent bottle are not empty, run the pumps to remove air bubbles
Poor resolution	Bad column	Change column
	Incorrectly seated column	Loosen column holder and reseal column
	Unstable pump pressure	Check for crimps in tubing and leaks in system and call Bio-Rad, make sure reagent bottle are not empty, run the pumps to remove air bubbles
	Incorrectly set temperature	Call Bio-Rad
	Blockage in system	Perform bleaching procedure, call Bio-Rad if needed
Unstable baseline		
Baseline is not smooth	Unstable pump pressure	Check for crimps in tubing and leaks in system and call Bio-Rad, make sure reagent bottle are not empty, run the pumps to remove air bubbles
	Air in pump A or B	Run the pumps with 0 –100% A or B
	Improper change of buffer bottle	Open pump inlet port and draw buffer until no bubbles are present
Baseline drifts up/down	Detector problem	Call Bio-Rad
	Max or min volts were changed	Change volts setting per protocol
Peaks outside of window		
Longer RT	Improperly seated column	Loosen column holder and reseal column

	Blockage in Autosampler	Perform bleaching procedure
	Leaks in injection valve	Look for drops on sample probe indicating a leak, call Bio-Rad
	Crimped tubing	Uncrimp if possible, call Bio-Rad
Shorter RT	Flow rate too fast	Set flow rate correctly
	Pump B overriding Pump A	Call Bio-Rad
	Incorrect column temperature	Call Bio-Rad

APPROVALS

Revised by: _____ Date: _____

Primrose Gulati & Meena Jain
Public Health Chemist Public Health Chemist

Reviewed by: _____ Date: _____

Faezeh Samimi
Supervising Chemist

Approved by: _____ Date: _____

George R. Helmer
Acting GDLB Chief

Revision Log

Enter section(s) and the page number(s) where deletion, revision, or add-ons are found. Indicate whether this is a deletion, revision, or an add-on by entering "X" in the appropriate column.

Procedure: Automated High Pressure Liquid Chromatography Screening Assay for Detection of Hemoglobins, Tracking #CN001, Version 5.2

Revised by: Primrose Gulati and Meena Jain

Date: February, 2013

Sections	Page #	Deletion	Revision	Add-on
IV.A.1a.1	2	<u>X</u>		
IV.B.1	3			X
VI.B	5			X
VII.D	15		X	
VII.N.1	28		X	
VII.N.6	29			X
VII.P.2.b	30		X	
VII.P.3.c	33		X	
VII.P.3.Q	34	<u>X</u>		

